

INVESTIGATIONS ON BILE PIGMENT BY ULTRA-
VIOLET SPECTROPHOTOMETRY

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA

BY

GEORGE EARL DAVIS

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

JUNE 1929
29



3781
M66D

VITA

AUTHOR—George Earl Davis.

BORN—Warsaw, Indiana, December 29, 1889.

ACADEMIC HISTORY—Graduated from the Warsaw, Indiana, High School in 1908. B.A. '14, Indiana University. M.S. '22, Iowa State College. Entered the Graduate School, University of Minnesota, in 1927; received the Ph.D. degree in June 1929, major Biophysics, minor Mathematics.

HONORS—Sigma Xi, Phi Kappa Phi.

PROFESSIONAL EXPERIENCE—Instructor in Physics, Chemistry, and Mathematics, Elwood, Indiana, High School, 1914-15, and in the Mishawaka, Indiana, High School, 1915-18. Fellow, Department of Physics, Rice Institute, 1919-20. Instructor in Physics, Iowa State College, 1920-25. Assistant Professor of Physics, Iowa State College, 1925-27. Assistant, Department of Biophysics, Mayo Clinic, summer 1926. Fellow in Biophysics, Mayo Foundation, 1927-29. Head of Department of Biophysics, Desert Sanatorium and Institute of Research, Tucson, Arizona, 1929-32. Visiting Research Associate, Department of Physics, Iowa State College, 1933. Research Fellow, Department of Physics, Iowa State College, 1933-34. Co-ordinator of Public Works, Iowa State Planning Board, 1934-35. Statistical Co-ordinator, Iowa State Planning Board, 1935-36. Assistant Professor of Physics, Duquesne University, 1936-37. Associate Professor of Physics, Duquesne University, 1937—.

PRESENT POSITION—Associate Professor of Physics, Duquesne University, Pittsburgh, Pennsylvania.

PUBLICATIONS—

Coefficients of Diffusion of Certain Alkali Salt Vapors in the Bunsen Flame. *Phys. Rev.*, 24:383-395. 1924.

The Spectrophotometric Determination of Hemoglobin. *Proceedings of the Weekly Staff Meetings of the Mayo Clinic*, 1:125-126. 1926.

The Spectrophotometric Determination of Hemoglobin (with C. Sheard). *Arch. Inter. Med.*, 40:226-236. 1927.

Investigations on Bile Pigment, Purified Bilirubin and Blood by Ultraviolet Spectrophotometry (with C. Sheard). *Am. Jour. Physiol.*, 90:515-516. 1929.

Ultraviolet Spectrophotometry: Comments on Apparatus and Experimental Methods (with C. Sheard). *Jour. Optical Soc. Am.*, 21:47-58. 1931.

Instruments for Radiometric and Radio-biological Investigations at the Desert Sanatorium and Institute of Research, Tucson, Arizona. *Gen. Elec. Rev.*, 34:98-103. 1931.

A Thermoelectric Thermometer for the Determination of Rectal Temperatures. *Arch. Physical Therapy, X-ray, Radium*, 12:724-730. 1931.

Twenty-nine Months of Solar Radiation at Tucson, Arizona (with J. L. McCarthy). *Monthly Weather Rev.*, 60:237-242. 1932.

Hemolysis and the Solar Spectrum (with W. W. Lepeschkin). *Protoplasma*, 20:189-194. 1933.

The Spectrophotometric Investigation and Determination of Bilirubin (with C. Sheard). *Jour. Lab. and Clin. Med.*, 19:593-608. 1934.

The Absorption Spectra of the Direct and Indirect Reacting Types of Serum Bilirubin. *Jour. Lab. Clin. Med.*,

Continued on inside back cover

Davis, George E., A.B., M.S., Ph.D., was born December 29, 1889, at Warsaw, Indiana; received the degree of A.B. in 1914 from Indiana University; attended the University of Chicago for one term in 1916, and Rice Institute, Houston, Texas, in 1919 and 1920; received the degree of M.S. in 1922 from Iowa State College; taught in high school for four years, and was instructor and assistant professor of physics at Iowa State College from 1920 to 1927. He entered The Mayo Foundation as a Fellow in Biophysics October 1, 1927. His services included biophysics, eighteen months and attendance at the University of Minnesota Medical School, three months. He received the degree of Ph.D. in 1929 from the University of Minnesota.

BIBLIOGRAPHY

- The spectrophotometric determination of hemoglobin. *Arch. Int. Med.* 40: 226-236 (Aug.) 1927 (with Charles Sheard); Abstr. in *Proc. Staff Meet. Mayo Clinic.* 1: 125-127 (Sept. 15) 1926.
- Ultraviolet spectrophotometry; comments on apparatus and experimental methods. *Jour. Optical Soc. Am.* 21: 47-58 (Jan.) 1931 (with Charles Sheard).
- Instruments for radiometric and radio-biological investigations at the Desert Sanatorium and Institute of Research, Tucson, Arizona. *Gen. Elec. Rev.* 34: 98-103, 1931.
- A thermoelectric thermometer for the determination of rectal temperatures. *Arch. Phys. Therap., X-ray, Radium.* 12: 724-730 (Dec.) 1931.
- Twenty-nine months of solar radiation at Tucson, Arizona. *Month. Weather Rev.* 60: 237-242, 1932 (with J. L. McCarthy).
- Hemolysis and solar spectrum. *Protoplasma.* 20: 189-194 (Nov.) 1933 (with W. W. Lepeschkin).
- The spectral distribution of erythema effectiveness of ultraviolet radiation. *Proc. Iowa Acad. Sc.* 40: 147, 1933.
- The spectrophotometric investigation and determination of bilirubin, with a consideration of oxyhemoglobin and its effect on the estimation of bilirubin. *Jour. Lab. and Clin. Med.* 19: 593-608 (March) 1934 (with Charles Sheard).
- Public works; a program of construction in Iowa. The second report of the Iowa State Planning Board, pp. 204-224 (April) 1935.

Reprinted from THE JOURNAL OF THE OPTICAL SOCIETY OF AMERICA
VOL. 21, No. 1, JANUARY, 1931

ULTRAVIOLET SPECTROPHOTOMETRY: COMMENTS ON
APPARATUS AND EXPERIMENTAL METHODS

BY

GEORGE E. DAVIS AND CHARLES SHEARD

3781
m66D

575126

ULTRAVIOLET SPECTROPHOTOMETRY: COMMENTS ON APPARATUS AND EXPERIMENTAL METHODS*

BY GEORGE E. DAVIS** AND CHARLES SHEARD

[DIVISION OF PHYSICS AND BIOPHYSICAL RESEARCH,
THE MAYO FOUNDATION, ROCHESTER, MINNESOTA

RECEIVED AUGUST 22, 1930

ABSTRACT

A simple and satisfactory type of container for the under-water spark, with suitable electrical circuits, and a new type of quartz cell to contain the liquids under examination are described. Some points are presented concerning the use of the under-water spark and the methods of obtaining data with the quartz spectrograph and Hilger rotating sector photometer. Desirable modifications of the usual apparatus are suggested in order to increase the rapidity of the method and enhance its field of usefulness, particularly in the study of unstable solutions with varying transmissive properties.

The increasing application of ultraviolet spectrophotometry to the problems of physics and chemistry^{1,2,3,4,5,6,7,8} has made it desirable to improve, wherever possible, the apparatus and methods in common use. It is our purpose to describe: (1) electrical circuits for the under-water spark which have been found satisfactory; (2) a container of new design for the under-water spark, and (3) a new type of quartz absorption cell for use in the examination of liquids. We shall call attention to certain important points concerning the design and use of the under-water spark, with the quartz spectrograph and Hilger rotating sector photometer, the methods of obtaining data from the photographic plates and desirable modifications of the apparatus to permit greater speed in obtaining the spectrograms. The new designs of apparatus were worked out during the course of certain experiments on bile pigment commenced during the latter part of 1927.

ELECTRICAL CIRCUITS FOR THE UNDER-WATER SPARK

The circuits to be described are similar to those employed by Howe,⁶ so far as general arrangement is concerned. But the details of construction and of operation may be of interest to those who wish to construct such circuits, since it is somewhat difficult to predict the results which an untried circuit will give with the under-water spark. The following

* Abridgment of a portion of thesis submitted by George E. Davis to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the degree of Ph.D. in Biophysics.

** Now at The Desert Sanatorium and Institute of Research, Tucson, Arizona.

circuits have been very satisfactory in connection with an under-water spark gap from 12 to 18 mm in length, used as source of radiation, with the quartz spectrograph and Hilger rotating sector photometer.

In Fig. 1, R is a 110 volt, 2.5 kilowatt Thordarson transformer,

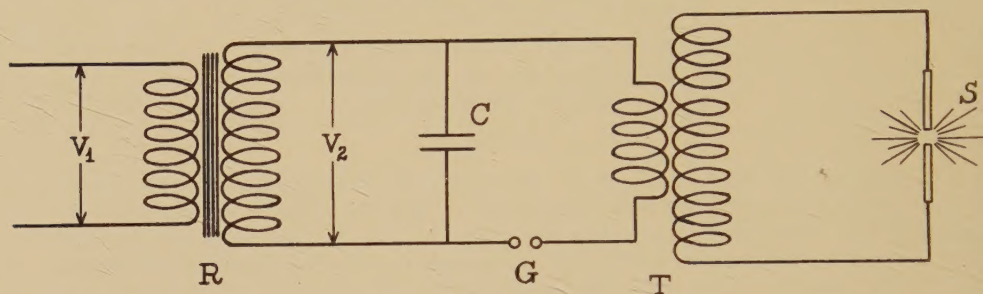


FIG. 1. *Electrical connections for under-water spark.*

supplied with 60-cycle current under a potential difference V_1 of about 97 volts. Current from the secondary, at about 17,600 volts V_2 , passes to capacity C and to the primary of the Tesla coil T . C consists of three Thordarson condensers in parallel, with a combined capacity of 0.027 microfarad. The condenser plates are of aluminum with rolled edges, immersed in oil and separated by thick mica plates. Discharge through the primary of T occurs at breakdown of the spark gap G . For the latter a radio quenched spark gap with 19 sections was used. As compared to the open gap, this type has the decided advantage of not producing objectionable gases (ozone and oxides of nitrogen). Also it is quiet and steady in operation and does not require an air blast or moving parts. Its breakdown potential can easily be varied by removing or inserting spark gap sections. The Tesla coil T was built according to suggestions made by Prof. R. C. Gibbs of Cornell University. The secondary consists of 180 turns of insulated No. 18 copper wire wound on a wooden core about 53 cm long and 29 cm in diameter. The primary is formed of 8 turns of heavy wire wound on a wooden ring 39 cm in diameter and 11 cm wide surrounding the secondary coil at its midpoint.

This arrangement provided ample electrical energy at the spark gap S . The voltage was sufficient to ensure a continuous succession of sparks with the terminals separated any distance to about 18 mm using distilled water of ordinary purity (single distillation). With greater separation of the terminals the spark refused to pass or was inconstant in action. Unless the water is fairly pure it is difficult to obtain a disruptive discharge with the terminals only a few millimeters apart.

UNDER-WATER SPARK GAP AND CONTAINER

Various types of containers were tried and discarded before a device was evolved that was satisfactory in every way. The design finally adopted is much simpler than the one described by McNicholas.⁹ Although the latter provides for somewhat more rapid adjustment of the length and position of the spark gap, such adjustments need be made only infrequently and can be carried out readily on the type of container we are to describe. This container has been in use for more than a year and has proved to be very satisfactory.

The construction is shown in detail in Fig. 2. The walls of the container are built of 0.5 in. sheets of hard rubber held together by screws.* The joints are made water-tight by strips of thin rubber dam (dental) placed between adjacent surfaces and fastened to one surface with rubber cement. The rubber dam was found to be more satisfactory than cement alone, the joints remaining perfectly water-tight after a long period of use (more than a year). Thicker rubber was used under the front plate of the box to avoid tearing the rubber when it was necessary to remove the front of the container.

The electrodes *T* are of tungsten, approximately 2 mm in diameter. They are brazed to brass rods *A* and *B*, with which electrical connections are made from the Tesla coil. These rods pass through hard rubber rods *C* and *D*, 2.2 cm in diameter and about 10.5 cm long. The tungsten electrode fits the hole closely from point *P* to the tapered end of *C* and of *D*. From *P* to the outer end, the hole is a little larger than the brass rod. The holes are filled with hot De Khotinsky cement before the metal rods are inserted, thus sealing the holes and holding the rods firmly in position. Leakage around the hard rubber rods is prevented by rings *R*, *R* with soft rubber gaskets underneath. The gaskets are set in circular grooves in the end plates and are forced against *C* and *D* by the screws which pass through the rings. This device holds *C* and *D* firmly in place and allows for ready adjustment of the length and vertical position of the spark gap. Special provision for lateral adjustment is unnecessary, as the entire apparatus with its support may easily be moved and, once in the proper position, permanently clamped to the table. The tungsten electrodes are set about 12 mm apart. With use they slowly waste away, so must be adjusted occasionally.

* While this thickness of wall proved satisfactory, it perhaps would be well to use thicker sheet in order to increase the factor of safety. A second container was built of 0.5 in. sheets of cloth-fabricated bakelite.

A window *W* of polished quartz about 1.5 mm thick covers a 10 by 24 mm opening in the front of the box. The window is held against a sheet of soft rubber by four screws with rubber washers. The screws pass through holes drilled through the quartz plate. This method of holding the window was found to be satisfactory, the soft rubber preventing the shock of the electrical discharge from breaking the thin sheet of quartz

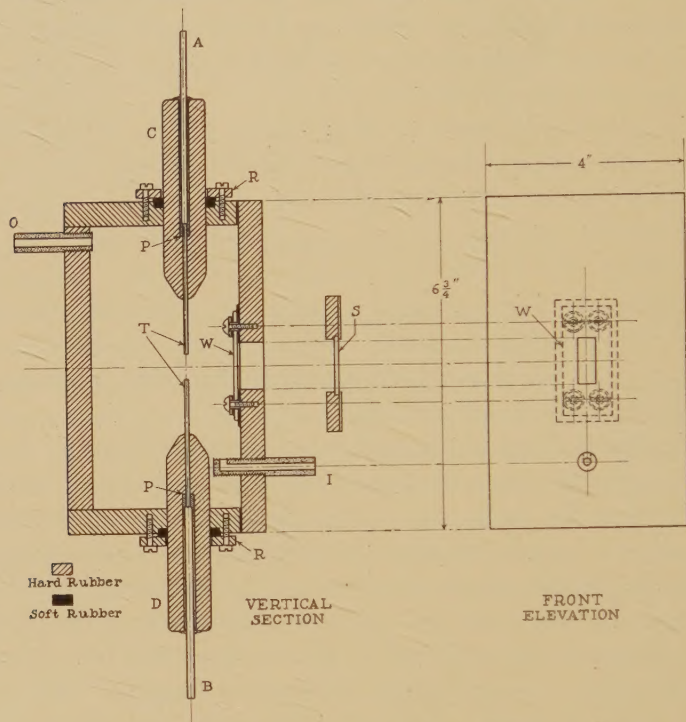


FIG. 2. Under-water spark gap and container.

even though the spark gap is within 2.5 cm of it. The window of another container, subsequently constructed, was held against the soft rubber by bone cleats at the sides. This device made it unnecessary to drill through the quartz plates. Whether it would prove satisfactory over a long period of use has not been determined. Radiation from the spark falls on a thin sheet of ground quartz *S*, which serves as a source of more uniform intensity than the spark itself. Without this diffusing screen the spectrograms were less uniform and satisfactory, even with the most careful alignment of the spark gap. The screen tends to smooth out the effect of occasional lack of uniformity occurring in the spark discharge.

Distilled water from an elevated reservoir of large capacity is led into the container at *I* and out at *O*. The outlet is so placed that an air cushion is left at the top of the container. This cushion serves to lessen the shock of the disruptive discharge. The flow of water may be regulated by a screw clamp on the rubber tube leading to the inlet. The water becomes impure with use and must be changed at regular intervals. With a spark gap of about 12 mm, a maximum of about fifteen photographic exposures of six seconds each can be made for each liter of water used.

Certain features in the design of the container deserve special mention. In an earlier model the electrodes were placed in the center of the container (a cylindrical one), the window was fastened to the outside surface of the wall, leaving a shallow recess on the inner side, and both inlet and outlet for water were located at the back. With this arrangement of parts the illumination of the screen *S* varied in distribution, so that the relative intensities of the upper and lower beams passing to the spectrograph were not constant. As a result it was impossible to trace the spectral transmission curves with certainty. It was observed that some of the small gas bubbles formed by the electrical discharge tended to collect at the quartz window, where they circulated about, apparently unable to escape readily from the shallow recess formed by the wall of the container. These bubbles were held to be chiefly responsible for the fluctuations in the light passing through the window. To prevent them from collecting, the window was mounted as shown in Fig. 2, so that no recess was formed on the inner side. At the same time the spark gap was moved nearer to the window so that the convection currents formed by the heated water and by the gas bubbles would tend to move upward past the window. Also, the inlet tube was moved to the front of the box so as to direct the stream upward past the window. With these changes in design the undesirable fluctuations of the light were eliminated and uniformly good results were obtained.

The container may be supported in any convenient manner while it is in use. In our work the container was fastened to a wooden panel sliding in vertical guides on a wooden support built somewhat like a box with front and top removed. The panel carried a millimeter scale at the edge to indicate vertical height and could be clamped in any position. The wooden support was clamped to the table which supported the spectrograph and sector photometer. This simple mounting for the spark gap container proved to be entirely adequate.

QUARTZ ABSORPTION CELL

Often it is desirable to examine thin films of liquid. The following construction of cell was found to be simple and satisfactory for films thin enough to be retained between quartz plates by capillarity.

Two rectangular quartz plates *Q*, Fig. 3, with accurately plane-polished faces were clamped together with a thin glass spacer *G* placed between them at one end. The plates measured 25 by 75 mm and were 1.5 mm thick. The glass spacer was a microscope cover glass measuring 25 by 40 mm and about 0.1 mm thick. For convenience in handling, it

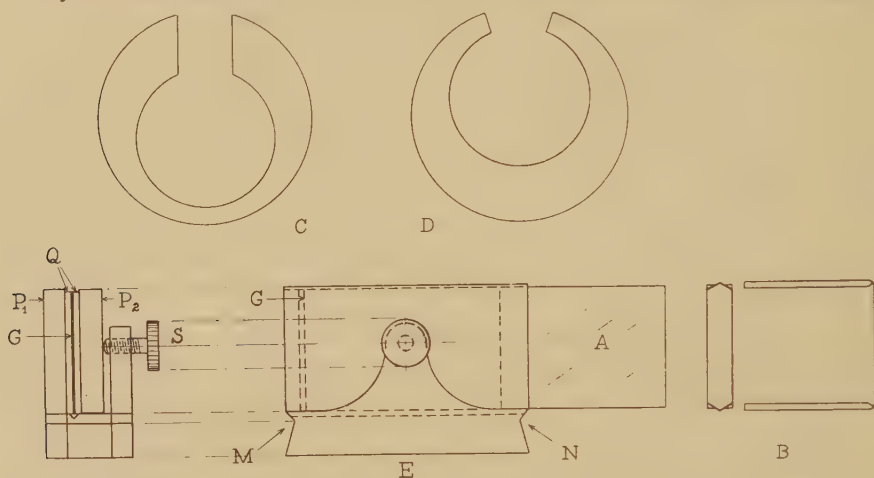


FIG. 3. Lower figures, construction of thin quartz cells. *C, D*, glass spacers for thicker cells.

was cemented to one of the quartz plates with a half-and-half mixture of beeswax and rosin so as to cover a little more than half of the plate. The clamp was formed by a fixed brass plate *P*₁ and a movable plate *P*₂ actuated by a screw *S*. The clamp with the quartz plates and spacer in position could be mounted quickly by inserting the edge *E* into a slot in a metal support, where it was held by a spring catch by means of the notches *M* and *N*. The liquid was held between the quartz plates at *A* by capillarity. If air bubbles were left between the plates when the liquid was run in, they were swept out by means of a fine thread, preferably of glass. Evaporation from the edges of the film was for the most part eliminated by slipping light aluminum covers over the edges of the slides. The covers were made by bending thin strips into the shape shown at *B*.

The thickness of the space between the free ends of the quartz plates was measured by means of a micrometer microscope. Readings were

taken at several points around the edge and averaged. In constructing a thin cell it should be remembered that the negative logarithm of the fraction of light transmitted is proportional to the thickness of transmitting substance. Therefore, if accurate relations between spectral transmission and thickness of solution are to be determined, variations in the thickness of the cell should be relatively small. Otherwise it is not allowable to take the average thickness as the effective thickness of the solution.

Thicker cells were made by clamping circular quartz plates (the circular shape is immaterial) against opposite sides of glass spacers shaped as shown at *C* and *D*. The quartz plates had parallel flat polished surfaces. The surfaces of the spacers were ground flat and parallel, then rubbed with a little paraffin and the excess scraped off. *C* is the spacer for the upper cell and *D* for the lower cell. Holders (not shown) were constructed of 1/8 inch brass plates. They consisted essentially of two leaves, one fixed and one hinged, between which the quartz plates, with glass spacer between, were held by the pressure exerted by springs passing from one leaf to the other. A circular opening was cut in each leaf, corresponding to that in the spacer, so that the radiation could pass through the cell. Each holder was provided with a flange like that shown at *E*, so as to fit into the same supporting device used for the thin cells.

NOTES ON EXPERIMENTAL METHOD

Accurate adjustment of the height of the under-water spark is important when this device is used with the quartz spectrograph* and rotating sector photometer. The correct height may be determined by making a series of test exposures with the quartz cells empty (or removed), changing the height of the spark by 0.5 mm steps. We found that even with such small changes in height, the corresponding successive changes in relative intensity of darkening of the adjacent spectrograms could easily be seen. Therefore it is evident that accurate vertical adjustment of the spark gap is essential to accurate results. It is felt that this point has not been fully appreciated in some of the quantitative measurements that have been made heretofore. The importance of accurate

* Reference is made to the usual form of spectrograph in which the absorption spectrum of the substance under examination is photographed in juxtaposition with the absorption spectrum of a comparison substance (the solute, in case of a solution) and points of equal intensity of darkening taken as corresponding to transmission ratios indicated by the sector photometer (or equivalent device). Quartz spectrograph E316, built by Adam Hilger, is such an instrument.

spark adjustment was pointed out by Howe, who used a horizontal spark gap. Substitution of the vertical gap has greatly lessened the difficulty of securing satisfactory spectrograms. But even with the vertical spark gap, accuracy of adjustment is of first importance.

Wasting away of the electrodes necessitates only occasional retesting of the height of the spark. In this respect the under-water spark has a decided advantage over the ordinary form of arc, since the terminals of the latter waste away much more rapidly.

The position (wave length) of a point of maximal or minimal transmission usually can be determined more accurately by examination of the photographic plate than by drawing the absorption curve which, from the positions of the plotted points, appears most probable in that region. It seldom happens that the transmission at a maximal or minimal point is close enough to any one of the predetermined values of transmission through the comparison substance to give the appearance of equal blackening of the adjacent spectrograms. Therefore, in general, we have two successive exposures, in one of which the spectrogram of the comparison substance is not dark enough for a match at the maximum or minimum, while in the other exposure it is too dark. In the one exposure we may locate the wave length at which the adjacent spectrograms most nearly match, while in the other we may find the wave length at which the spectrograms appear to differ most. With the under-water spark as source of radiation and using the usual type of photographic plate, these two observed values of wave length ordinarily are not the same, especially when the transmission or absorption band is broad and not especially prominent. Therefore, to estimate the true position of the maximum or minimum, both values should be observed and averaged, weighting the values according to the nearness of the transmission value to that of each of the two adjacent comparison spectrograms. The two observed values usually are not the same because of the fact that the intensity of blackening of the spectrograms, unmodified by absorbing substances placed in the paths of the beams of light, increases as one passes from the region of short wave length to that of the longer wave lengths, except in one region of the visible spectrum. Because of this, differences in intensity of darkening in adjacent spectrograms are more noticeable in the shorter wave lengths, and in general the effect is to cause points of greatest contrast to appear to be shifted toward the shorter wave lengths while points of greatest similarity appear shifted in the opposite direction.

It is necessary frequently to estimate values of transmission at points at which equal darkening of the adjacent spectrograms does not occur. This is usually the case at maxima and minima. Often at other points the shape of the curve is not well defined except by supplementing the values of transmission at match points by values estimated at other positions. In making such estimations it must be remembered that equal contrasts on the photographic plate do not necessarily represent equal differences in intensity of illumination. The slope of the intensity-darkening curve decreases as the intensity increases, at least above very small intensity values. This should be taken into account in comparing the darkening of the spectrogram in question with that of each of the adjacent comparison spectrograms, next lower and next higher in transmission, especially when the steps between successive intensities of the comparison spectrograms are large. Obviously in making such comparisons it is advantageous to have the darkening of the comparison spectrograms increase or decrease regularly as the transmission of the variable sectors of the photometer is made progressively larger or smaller. This will be the case if only the size of the sector openings is varied from one exposure to the next. Therefore the spark should be operated for the same length of time for all the exposures. For the same reason the spark discharge should be kept as nearly as possible an unvarying source of radiation throughout the series of exposures.

Many solutions undergo rapid chemical or physical changes on standing or on exposure to certain agencies such as heat and radiation. Clearly the absorption curve obtained for such a solution cannot be correct unless the time required for obtaining the data is less than that required for the occurrence of noticeable changes in the transmissive properties. Undoubtedly great scientific value would attach to any method which would enable us to obtain correct absorption curves for such solutions.* Such a method would make it possible to follow the chemical or physical changes by means of the corresponding progressive changes in the absorption spectra. Observations of this nature would be extremely useful in the study of biological fluids.

* A rapid recording spectrophotometer for measuring the color of a reflecting surface has been designed. See A. C. Hardy, *A Recording Photoelectric Color Analyser*, J.O.S.A. and R.S.I., 18, 96-117; 1929. Hardy's apparatus may be used for measurements on transmission in the visible spectrum as well as for reflection. Rapidly operating photoelectric spectrophotometers are described by Ellsworth and others, *Oil and Fat Industries*, 7, 15-19; 1930, and by Mulder and Rasek, J.O.S.A., 20, 155-156; 1930, and *Textile World*, February, 1930.

In spite of its high accuracy, and other commendable features, the quartz spectrograph (of the usual type represented by the Hilger spectrograph E316) with rotating sector photometer is wholly unable to cope with rapid changes in the transmissive properties of the substance under examination. As a rule, about sixteen exposures are required for the accurate determination of an absorption curve, and frequently twice that number are required. Under the best working conditions, a minimal time of about eight minutes is required for sixteen exposures (on a single plate) or eighteen minutes for thirty-two exposures (on two plates). This precludes the possibility of obtaining the absorption curve of a solution which is rapidly fading or otherwise changing in its ability to transmit radiation.

However, it should be possible to modify the apparatus so as to allow much greater speed in obtaining data by this method without sacrifice of accuracy. Of the total amount of time required for obtaining a series of spectrograms, by far the greater part is used in adjusting the photometer sectors and the plate holder. With the under-water spark and accessories described in this paper, five or six seconds of spark operation was found to be sufficient for a single photographic exposure, except for values of transmission as low as 1 or 2 percent.* Considering all transmission values from 1 to 100 percent and with an intense under-water spark as source of radiation, the average time of spark operation for a single exposure need not exceed five seconds. This time is small compared to that required for resetting the sector photometer and plate holder, for these operations require twenty-five to thirty seconds under the best conditions. Therefore it is evident that we can increase the speed of the method most effectively by reducing the time required for the necessary adjustments of the photometer and plate holder. It is suggested that these adjustments should be made automatic. In that which follows we shall describe briefly the essential features which seem desirable in such a mechanism and shall suggest some suitable details of construction.

The photometer sectors should be capable of accurate adjustment while in motion, as are those of certain photometers now in use. Settings should be controlled by a series of stops whose positions can be prearranged. These stops may be released in their proper order electro-

* A considerable range in intensity of darkening of the comparison spectrograms (beam through solvent) results if the same time of spark operation is used for all transmission values, of course. Usually this presents no difficulties and frequently less time is required than in attempting to secure equal blackening of all the comparison spectrograms.

magnetically or by a clock mechanism. In case the photometer settings are made by rotating a graduated head, a constant torque may be applied to the head by a motor-driven friction disk, so that when a stop is released the head is turned to its next position.

It should be unnecessary to change photographic plates while obtaining data for a single absorption curve, whether many or few exposures are required. Therefore both wide and narrow plates should be available, with plate holder (or holders) built to accommodate the various widths. If plates wide enough to accommodate thirty or more exposures are objectionable, it should be possible to lay two or more narrow plates side by side in the same plate holder.

The plate holder should be so mounted as to move easily and without "play" under the action of its own weight. Its settings may then be controlled by a series of stops in prearranged positions, the stops being released in consecutive order by a clock mechanism or electromagnetically.

A shutter should be arranged so as to close automatically and interrupt the exposure during resettings of the sector photometer and plate holder. Or the same effect should be accomplished by the automatic breaking of the current supply to the source of radiation.

With automatic adjustments operating essentially as described, it seems that each photographic exposure and subsequent resetting of the plate holder and photometer could be accomplished in eight seconds or less. Thus the time required to obtain sixteen exposures for an absorption curve would be reduced from about eight or nine minutes to approximately two minutes.

If only a single absorption curve is to be determined, exposure of the wave-length scale (or standard line spectrum) can be made at leisure after the run. But if a series of curves is required, as in following the chemical or physical changes of an unstable solution, exposure of the wave-length scale must be made before changing plates. With proper provision for rapid plate changing, it should be possible to make two exposures of the wave-length scale and change plates in thirty seconds or less. Thus for a series of absorption curves for the same specimen, about half a minute must be added for these operations, making the time for each curve about two and a half minutes (sixteen exposures) or four and a half minutes (thirty-two exposures). The reductions in time here represented would greatly enlarge the field of applicability of the method, particularly along the lines we have indicated, without any sacrifice of the high accuracy now attainable.

BIBLIOGRAPHY

1. Daminovich, Horacio and A. T. Williams: *Ultraviolet spectrophotometry as a method of examining the mechanism of certain chemical reactions, and as a method of qualitative and quantitative determination*. *Anales soc. cient. Argentina*, 98, 241-274; 1924.
2. Baly, E. C. C., R. A. Morton and R. W. Riding: *Measurement of absorptive power*. *Proc. Roy. Soc. A*, 113, 709-716; 1927.
3. Gibbs, R. C. and C. V. Shapiro: *The absorption spectra of some halogenated fluoresceins*. *J. Amer. Chem. Soc.*, 51, 1769-1773; 1929. *The absorption spectra of some phthaleins of the trihydroxyphenols*. *J. Amer. Chem. Soc.*, 51, 1755-1766; 1929.
4. Gibson, K. S., H. J. McNicholas, E. P. T. Tyndall, M. K. Frehofer and W. E. Matthewson: *Spectral transmissive properties of dyes*. Bureau of Standards, *Sci. Paper*. No. 440, 121-184; 1922.
5. Hicks, C. S.: *Relationship of thyroxin to tryptophan*. *J. Chem. Soc.*, 127, 771-776 1925. *Tautomerism in the thyroxin molecule*. *J. Chem. Soc.* 128, 643-645; 1926.
6. Howe, H. E.: *On a modification of the Hilger sector photometer for measuring ultraviolet absorption and its application in the case of certain derivatives of fluoran*. *Phys. Rev. Series II*, 8, 674-688; 1916. *Vide later articles in Physical Review*.
7. Pool, G. M.: *The measurement of absorption in the ultraviolet spectrum*. *Z. Physik*, 29, 311-314; 1924.
8. Stenstrom, Wilhelm and Melvin Reinhard: *The influence of the pH upon the ultraviolet absorption spectra of certain cyclic compounds*. *J. Phys. Chem.* 29, 1477-1481; 1925. *Ultraviolet absorption spectra of blood serum and certain amino acids*. *J. Biol. Chem.* 66, 819-728 1926.
9. McNicholas, H. J.: *Use of the under-water spark with the Hilger sector photometer in ultraviolet spectrophotometry*. *Bur. Stand. Jour. Res.*, vol. 1, December, No. 6, 939-949; 1928.

THE SPECTROPHOTOMETRIC INVESTIGATION AND DETERMINATION OF BILIRUBIN*

WITH A CONSIDERATION OF OXYHEMOGLOBIN AND ITS EFFECT ON THE
ESTIMATION OF BILIRUBIN

GEORGE E. DAVIS, PH.D.,† AND CHARLES SHEARD, PH.D., ROCHESTER, MINN.

INTRODUCTION

VISUAL spectrophotometry has been employed in a number of investigations on bilirubin. In 1925 and 1926, Mann, Sheard, Bollman and Baldes¹³⁻¹⁷ obtained extensive spectrophotometric data bearing on the question of the sites of formation of bilirubin, the relative amounts of bilirubin formed at the different sites, and the formation of the pigment from hemoglobin. In 1926 and 1927, Bollman, Sheard and Mann investigated the absorption of bile pigment from the intestine² and the bilirubinemia following extirpation of the gall-bladder and ligation of the common bile duct.³ Spectrophotometric comparisons of the nature and amount of bile pigment found in the blood serum under normal and pathologic conditions were made by Magath and Sheard¹² in 1927. The effect of intravenous injections of chlorophyll on the rate of formation of bilirubin was investigated by Bollman, Sheard and Mann.⁴ In these researches the spectrophotometric data were obtained by means of a Keuffel and Esser "color analyzer," which allows transmissions to be determined visually in a spectral range extending approximately to wave length 430 $m\mu$ (millimicrons).

Various data and conclusions concerning the spectrophotometry of bilirubin have been published by Sheard, Baldes, Mann and Bollman.¹⁹ This work will be referred to later. Sheard, Mann and Bollman²⁰ have made spectrophotometric comparisons of purified bilirubin and the yellow pigment (bilirubin) found in bile and in blood serum and plasma. In both of these investigations fading of the pigment is demonstrated, the effects of hemolysis and turbidity are described and discussed, and the laws of Lambert and of Beer are shown to be applicable to solutions of the pigment. These investigations have amply demonstrated the sensitivity, accuracy and practicability of the spectrophotometric method of investigating bilirubin.

In this paper we shall present and discuss the applications of spectrophotometry to investigations on the bilirubin in the blood and in other body fluids. In addition we shall present some observations and experimental results concerning spectrophotometric determinations of oxyhemoglobin and the

*From the Division of Physics and Biophysical Research, the Mayo Foundation.

Abridgment of a portion of thesis submitted by George E. Davis to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the degree of Ph.D. in Biophysics.

Received for publication April 6, 1933.

†Desert Sanatorium and Institute of Research, Tucson, Arizona.

effects of the presence of small amounts of this pigment on the quantitative determination of bilirubin.

The experimental data on which this article is based were obtained during the course of various investigations on bile pigment, concerning which a report will be made later. Our spectrophotometric observations are an extension of those made by Sheard and coworkers, to which reference has been made.

SPECTROPHOTOMETRIC THEORY AND ITS APPLICATIONS

To simplify the discussion we first review briefly some important principles of spectrophotometry and discuss their applications.

Consider a beam of light passing through a layer of solution of a substance X which is under investigation. Let I represent the intensity of the transmitted beam and I_0 the intensity which the beam would have had if it had been absorbed (in part) only by the solvent. Then the fractional part transmitted by X alone is $\frac{I}{I_0}$. Call this T, the transmission of X. Let C, L, ϵ and A represent the concentration, the thickness, the extinction coefficient (or absorption coefficient) and the absorption ratio, respectively. By definition, ϵ is the reciprocal of that thickness for which $T = 0.1$, while $A = \frac{C}{\epsilon}$, a constant.

It follows from Bouguer's law (commonly called Lambert's law) that

$$C = -\frac{A}{L} \log_{10} T. \quad - \quad - \quad - \quad - \quad - \quad - \quad - \quad - \quad - \quad - \quad (1)$$

If the value of A is known, this relation gives the concentration C upon substituting measured values of L and T. The expression $-\log_{10} T$ represents the optical density of X when measured in thickness L and concentration C.

It is easily shown from Equation (1) that for any one substance the ratio of the optical densities corresponding to any two wave lengths in the spectrum is a constant, regardless of the value of C or of L. Therefore, since no two substances have exactly the same transmission curve, a substance can be identified by showing that at various wave lengths the optical densities have the relative values known to be correct for that substance. Or, conversely, the entire transmission (or absorption) curve of a substance in solution can be calculated from the known values of the density ratios for the substance, if we know the value of T at some one wave length or can find it from the value of A and Equation (1).

In order that spectrophotometric observations on a solution may reveal only the absorption due to the dissolved substance, the absorption in the solvent, if not negligible, usually is balanced out by filling the comparison cell with the solvent. In physiologic fluids there are, in general, other substances in solution (or in colloidal suspension) besides the substance under investigation. All such substances absorb radiation; and since in many cases the absorption is not negligible, it is best, if possible, to balance it out by using as comparison fluid the identical physiological fluid, similarly diluted, with only the substance removed of which the transmission is to be measured.* If the substance cannot

*We usually neglect the error due to absorption in that part of the fluid which, in the comparison cell, occupies the space occupied in the other cell by the substance under investigation. In most cases the error is small.

be removed without altering the physical or chemical properties of the fluid, some fluid, such as distilled water, having negligible absorption, may be used as comparison substance. Correction must then be made for any appreciable absorption due to each of the other substances in the physiological fluid. The concentration of each substance must be found by some independent method and the corresponding transmission at each wave length computed by means of Equation (1). The transmission of the substance under investigation may then be found at any given wave length by dividing the observed transmission by the product of the transmissions of the other substances. In investigating bile pigment we have used this method in correcting for the absorption due to traces of oxyhemoglobin.

APPARATUS AND EXPERIMENTAL METHOD

The spectrophotometric observations were made by means of a quartz spectrograph and Hilger rotating sector photometer, with an under-water spark between tungsten terminals as source of radiation. These instruments were assembled and used in the usual manner, following essentially the method first described by Howe.⁶ The electrical circuits, quartz cells and container for the under-water spark have been described elsewhere.⁷

While the quartz spectrograph is to be preferred in most investigations because of its high dependability, our discussion of principles and methods will be understood to be applicable to the spectrophotometric investigation of bilirubin regardless of the kind of apparatus employed in finding the spectral transmission or absorption.

INTERFERENCE OF OXYHEMOGLOBIN WITH SPECTROPHOTOMETRIC OBSERVATIONS ON BILIRUBIN

It was found by spectroscopic examination that the majority of blood serums, however carefully prepared, contain traces of hemoglobin in the form of oxyhemoglobin, sufficient in amount to affect the absorption spectrum appreciably, even though the unaided eye may be able to detect little or none of the characteristic red color. No method is known whereby bilirubin alone can be removed from blood serum with the assurance that the serum will remain unchanged chemically or physically. This fact precludes the possibility of balancing out the absorption due to oxyhemoglobin (and other substances) by using the bilirubin-free serum as comparison fluid. Therefore in spectrophotometric measurements on bilirubin, corrections must be made for the radiation absorbed by oxyhemoglobin. The importance of this has been pointed out by Mann, Sheard, Bollman and Baldes¹⁴ in connection with visual spectrophotometric observations to determine the site of formation of bilirubin. Oxyhemoglobin has a very strong absorption band at the short-wave end of the visible spectrum, in the region covered by the most prominent and characteristic absorption band of bilirubin. The maximal optical density in the latter band is roughly twelve times as great as in the former. But we have found that the concentration of oxyhemoglobin in a serum which appears to the unaided eye to be free from red color actually may be much greater than the concentration of bilirubin; so that

the absorption in the oxyhemoglobin may be comparable to that in the bilirubin, in spite of the greater power of absorption of the latter.

To test the visibility of traces of oxyhemoglobin, various concentrations in water were prepared and the color noted when viewed against an illuminated white background. Thicknesses of solution of 1.5 and 9.0 cm. were examined, making direct comparisons with pure water. Data for the higher dilutions are given in Table I. Visual detection of oxyhemoglobin in blood serum is much

TABLE I
VISUAL APPEARANCE OF AQUEOUS SOLUTIONS OF OXYHEMOGLOBIN

HEMOGLOBIN, CONCENTRATION IN MG. PER C.C.	COLOR, VISUALLY OBSERVED THROUGH 1.5 CM. DEPTH	COLOR, VISUALLY OBSERVED THROUGH 9.0 CM. DEPTH
0.036	Not detectable, except when compared with pure water	Very faint pink, but noticeable
0.024	Not easily detected, even when compared with pure water	Hardly detectable, except when compared with pure water
0.007	Not detectable	Not detectable.

more difficult than in water, since the color of the serum tends to mask that of the oxyhemoglobin. We have found by spectroscopic and spectrophotometric measurements that amounts of from 0.05 to 0.10 mg. per c.c. frequently escaped detection by the unaided eye; while in one case a serum containing approximately 0.2 mg. of hemoglobin per c.c., or about 100 times the normal bilirubin content, appeared free from hemoglobin, the color being masked by that due to a high concentration of bilirubin. These concentrations of hemoglobin are easily high enough to modify the absorption spectrum greatly.

These data show clearly that in spectrophotometric studies of bilirubin in blood serum the possibility of errors caused by traces of oxyhemoglobin cannot be ignored, even in serums which appear free from red color. Corrections must be made for the effects of the oxyhemoglobin unless these effects can be shown to be negligible.

DETERMINATION OF CONCENTRATION OF TRACES OF OXYHEMOGLOBIN

The concentration of hemoglobin in traces of oxyhemoglobin in the serum was measured with a Keuffel and Esser spectrophotometer ("color analyzer") by the method described by the authors.⁶ This method, while accurate to 1 or 2 per cent for the concentrations of hemoglobin usually found in blood, is much less accurate for very low concentrations. But the accuracy is sufficient to permit of fairly reliable corrections for absorption by traces of oxyhemoglobin.

Another method, of about equal reliability for very low concentrations of hemoglobin, was used on some of the serums. The serum was diluted a known amount, shaken with air, and examined in a glass cell of adjustable thickness placed in front of the slit of a spectrometer. With light from an incandescant lamp (always the same lamp and voltage) passing through the cell, the thickness of serum was reduced until the absorption band of oxyhemoglobin at wave length 577 m μ disappeared. From the thickness at disappearance of the band, the concentration of hemoglobin was found by comparison with values obtained for known concentrations.

ABSORPTION SPECTRUM OF BILIRUBIN

To determine concentrations of bilirubin spectrophotometrically, it is necessary to measure the transmission only at one suitable wave length for which the absorption ratio is known. But in order to identify or otherwise investigate the pigment, one usually must determine the curve which represents the absorption spectrum. This may be represented by plotting absorptions, extinction coefficients or absorption ratios. Or transmissions may be plotted, since the values of transmission and of absorption are complementary. We shall plot transmissions.

So far as possible we shall reserve for a separate paper the discussion of the absorption spectra we have determined for various samples of bilirubin.

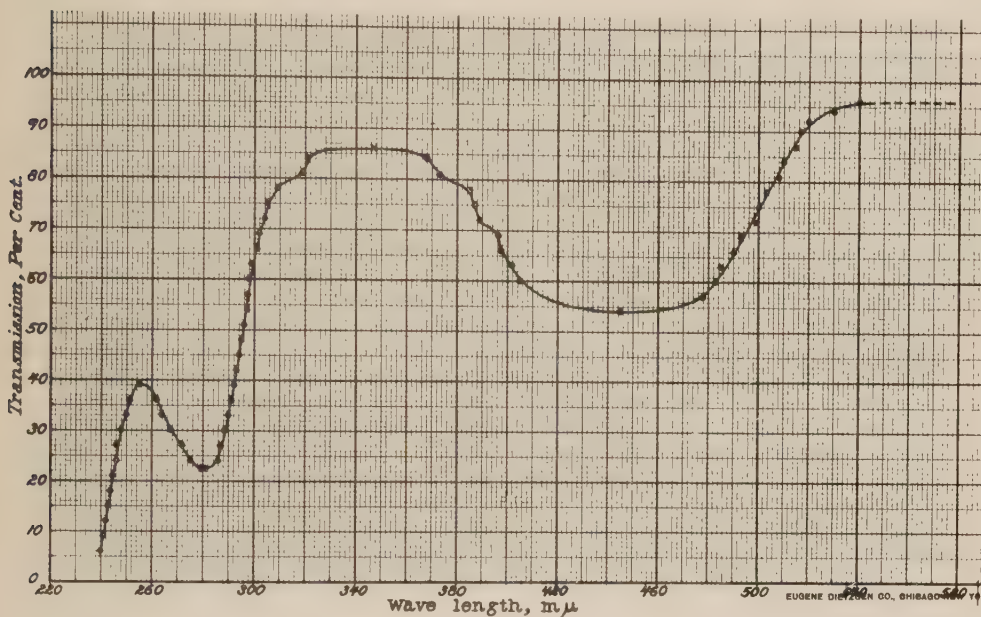


Fig. 1.—Spectrum of blood serum of jaundiced human being. For wave lengths longer than about 340 $m\mu$, the spectrum is typical of serum bilirubin. Concentration of bilirubin, 18.6 mg. per 100 c.c. Serum undiluted. Thickness, 0.128 mm.

However, to illustrate the general characteristics of the spectrum, we show in Figs. 1 and 2 the curves obtained for blood serums from two jaundiced patients. Distilled water was used as comparison substance, since bilirubin-free serum was not available, for reasons already stated. Hence the curves must show the absorption due not only to bilirubin but also to other substances in the serum. However, at wave lengths longer than 330 or 340 $m\mu$ no substance ordinarily present in serum, excepting hemoglobin or its compounds, is at all comparable to bilirubin in its power to absorb radiation. Therefore above 330 or 340 $m\mu$ the curves represent the absorption spectrum of bilirubin fairly closely, particularly in Fig. 1, where the concentration is very high. In Fig. 2 correction was made for the absorption due to oxyhemoglobin (upper curve), the broken line curve representing the corrected bilirubin spectrum. The curve in Fig. 1 needed no correction, since no oxyhemoglobin appeared in the serum.

The prominent band with maximal absorption at $280\text{ m}\mu$ is not characteristic of bilirubin. This is shown by the curves we have obtained for various samples of serum, from which it appears that the absorption in this region is independent of the concentration of bilirubin. Lewis¹¹ found the same band in the spectra of pseudoglobulin, euglobulin and albumin, from which he concluded that its appearance in the spectrum of blood serum is due specifically to the serum proteins. Later Stenström and Reinhard^{21, 22} decided from spectrophotometric evidence that the tryptophane and tyrosine constituents of the protein molecule are responsible for this band.

The curves in Figs. 1 and 2 are alike in general shape but differ in minor details. For the most part the minor differences are not due to experimental

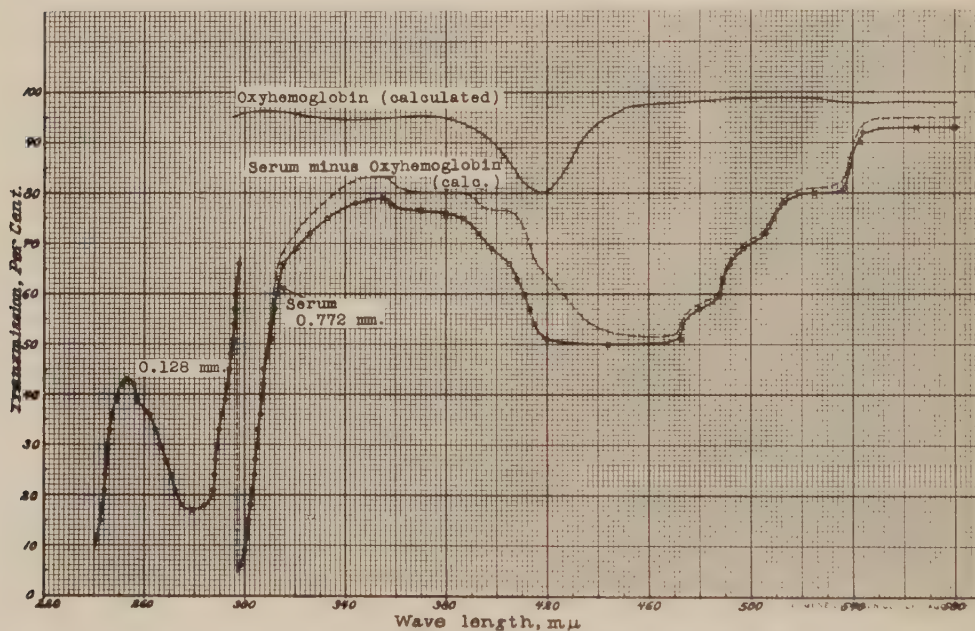


Fig. 2.—Spectrum of blood serum of jaundiced human being. For wave lengths longer than about $340\text{ m}\mu$, the general shape of the curve (broken line) is typical of serum bilirubin. The lower curve represents observed transmissions. The upper curve is calculated, to represent the trace of oxyhemoglobin present in the serum and is used in obtaining the corrected curve (broken line). Bilirubin concentration, $4.3\text{ mg. per }100\text{ c.c.}$ Serum undiluted. Thickness, above $298\text{ m}\mu$, 0.772 mm.

errors. The prominent feature of both curves, definitely characteristic of bilirubin, is the broad absorption band extending from the green region of the visible spectrum to the neighborhood of wave length $360\text{ m}\mu$ in the ultraviolet. The wave length of maximal absorption seems to vary somewhat. It is difficult to locate accurately, since the absorption band is very broad and rather “flat” at its lower end. In Figs. 1 and 2 maximal absorption is approximately at wave lengths 445 and $460\text{ m}\mu$, respectively. Practically all our curves for bilirubin in human blood serum gave evidence of maximal absorption at some wave length between these two values, the average being about $453\text{ m}\mu$. Rahier¹⁸ located it at $435\text{ m}\mu$ but did not state whether his observations were made on serum bilirubin or on solutions of purified bilirubin. We have found an aver-

age value of 432 $m\mu$ for purified bilirubin dissolved in 0.02 N sodium hydroxide, this value being the average of nine determinations.

ABSORPTION SPECTRUM OF OXYHEMOGLOBIN

The absorption spectrum of oxyhemoglobin, Fig. 3, was obtained from samples of blood from three normal men. Clotting was prevented by adding a little dry ammonium oxalate. The hemoglobin content was measured by the spectrophotometric method of the authors⁶ and was found to be normal in each case. Part of each sample of blood was centrifuged and the plasma used as fluid for comparison. The whole blood and plasma were diluted equally with 0.1 per cent sodium carbonate solution. After dilution the concentration of hemoglobin was 1.22 mg. per c.c. This solution was shaken with air to convert the

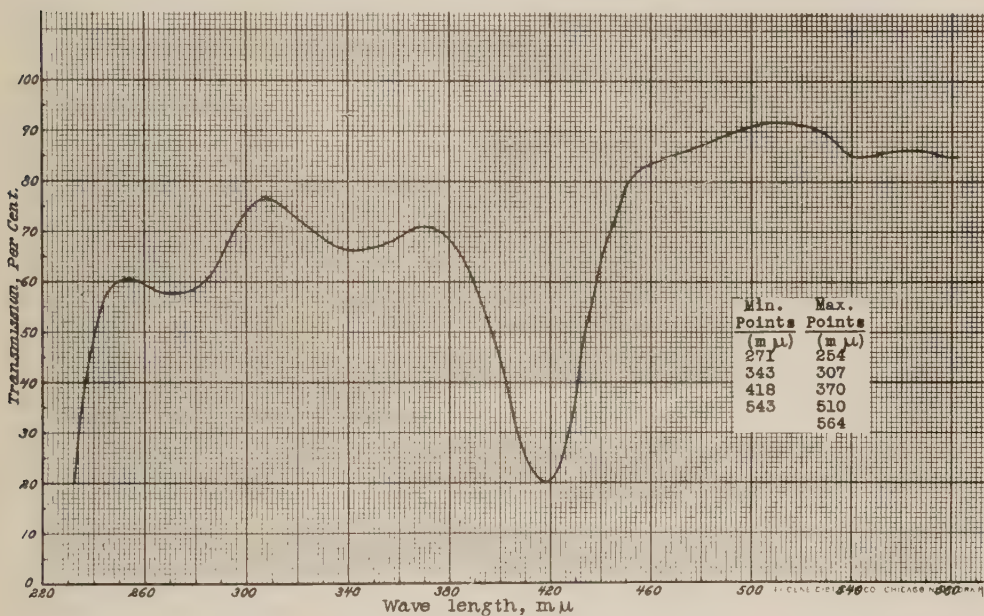


Fig. 3.—Spectrum of oxyhemoglobin normally present in human blood serum, computed for an assumed transmission of 30 per cent at wave length 427 $m\mu$.

hemoglobin into oxyhemoglobin. A layer 0.772 mm. thick was examined in each case.

Since the diluted blood was not centrifuged, the absorption represented in Fig. 3 includes that due to the stroma of the hemolyzed cells. The absorption by these cell bodies is small compared to that of the oxyhemoglobin, however. The following facts justify this statement: (1) Curves obtained by Strub²³ show this to be the case in the spectral range between wave lengths 450 and 660 $m\mu$. (2) In direct spectrophotometric comparisons of blood serums we have obtained several curves representing the spectrum of oxyhemoglobin alone. These agree closely with the curve in Fig. 3, from wave length 320 or 340 to 580 $m\mu$.

The curves from the three blood samples were exactly alike in general shape. Corresponding transmissions differed by less than the experimental

errors of measurement, over a major part of the spectral range represented. Positions of maxima and minima, 9 in all, agreed on the average to within 0.7 $m\mu$.

To calculate the curve of Fig. 3, a wave length in the principal absorption band was first chosen, at which wave length, 427 $m\mu$, all three curves indicated the same transmission. The optical densities ($-\log_{10} T$) at a considerable number of other wave lengths were each divided by the density at 427 $m\mu$ and at each wave length the average value of the three density ratios was used in calculating the transmission, arbitrarily assuming a value of 30 per cent at 427 $m\mu$. This procedure is based on the fact, previously stated, that for any two

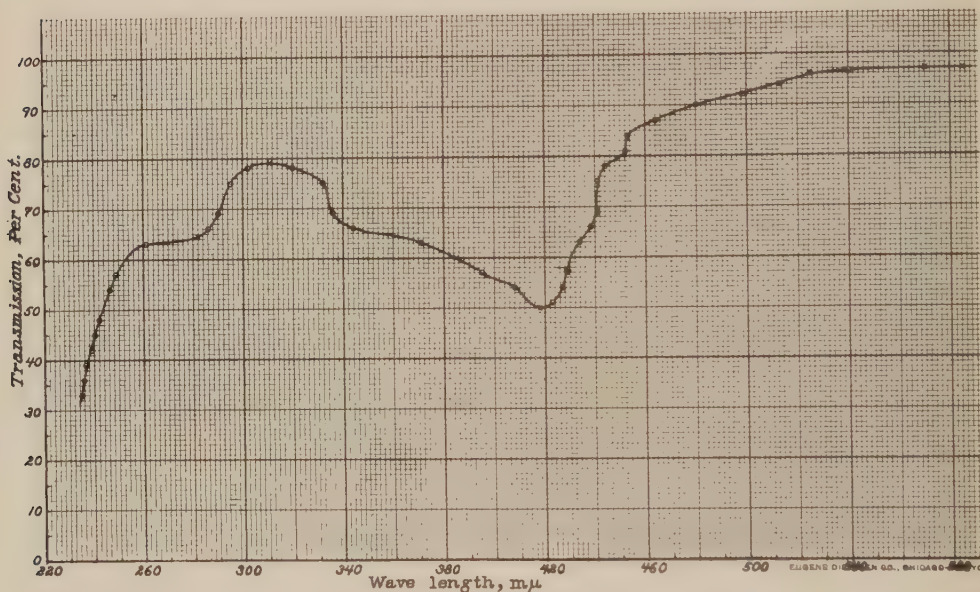


Fig. 4.—Spectrum of oxygenated solution of purified hemoglobin dissolved in water containing a little ammonium hydroxide. Concentration of hemoglobin, 0.00121 gm. per c.c. Thickness, 0.772 mm. The α - and β -bands, at 577 and 542 $m\mu$, respectively, were visually observed but are not shown here because of their faintness and the uncertainty in measuring transmissions near 100 per cent in this spectral region.

wave lengths the ratio of optical densities is a constant independent of concentration and thickness. Some of the average density ratios are shown in Table II.

For comparison with Fig. 3 we show in Fig. 4 the transmission curve for a solution of purified hemoglobin in water containing a little ammonium hydroxide (1 part of fairly concentrated ammonium hydroxide to 125 parts of water). The hemoglobin was prepared by the method of Welker and Williamson. In the solution the concentration of hemoglobin was 1.21 mg. per c.c. The solution was examined spectrophotometrically within thirty minutes after preparation and was not exposed to radiation which might cause it to "fade." The solution was shaken with air just before examination. We are not prepared to say whether the resulting compound of hemoglobin was oxyhemoglobin or a salt of hemoglobin. However, we note that when sodium hydroxide was substituted for the ammonium hydroxide, visual spectroscopic examination revealed a broad

TABLE II

SOME OF THE DENSITY RATIOS OF OXYHEMOGLOBIN USED IN COMPUTING THE CURVE OF FIG. 3

WAVE LENGTH λ	DENSITY RATIO ($= -\text{LOG}_{10} T_{\lambda} / -\text{LOG}_{10} T_{427}$) [*]
(m μ)	
235	0.896
245	0.460
254	0.416
270	0.459
290	0.360
307	0.221
320	0.267
340	0.339
360	0.310
370	0.284
380	0.315
390	0.430
400	0.664
405	0.893
410	1.15
418	1.33
427	1.000
433	0.606
442	0.328
450	0.194
460	0.151
480	0.114
510	0.071
525	0.081
542	0.136
565	0.122
580	0.135

^{*}Numerator and denominator represent the optical densities at wave lengths λ and 427 m μ , respectively.

absorption band centered at about wave length 620 m μ , in addition to the usual α - and β -bands at 577 and 542 m μ , shown by both solutions. The α - and β -bands were less distinct than in the solution with ammonium hydroxide. (In Fig. 4 these bands are not indicated, for reasons noted under the figure.) These differences show that the nature of the compound of hemoglobin obtained in such a solution depends on the kind of alkali which is added.

It is seen by comparing Figs. 3 and 4 that although the two spectra are generally similar, the spectrum in Fig. 4 differs from that in Fig. 3 in the following particulars: (1) considerably greater absorption in the spectral region lying approximately between wave lengths 340 and 395 m μ ; (2) very much less absorption in the principal part of the most prominent absorption band, roughly from 395 to 440 m μ ; (3) less absorption in the region of the α - and β -bands, 577 and 542 m μ . These differences could not be due to the stroma in the serum, since these bodies would increase rather than decrease the absorption shown in Fig. 3, which action could not cause the first difference, and since the stroma does not absorb specifically in the spectral regions of the three bands mentioned in the statement of the second and third differences. A reliable quantitative comparison of the two curves may be made by comparing corresponding ratios of optical density. Some such ratios with respect to the density at wave length 460 m μ are shown in Table III. Wave lengths of maximal and minimal absorption up to 510 m μ are represented, with some inter-

mediate values. While at each wave length the magnitude and algebraic sign of the difference shown in the last column are dependent on the choice of wave length at which the optical density is taken as reference, the great range in magnitudes and the wide departures from zero show without ambiguity that the two spectra cannot belong to identical substances, although the substances evidently are rather closely related chemically. This would be true also if sodium hydroxide were substituted for the ammonium hydroxide in preparing

TABLE III
COMPARISON OF DENSITY RATIOS FOR SPECTRA OF FIGS. 3 AND 4

WAVE LENGTH λ ($m\mu$)	DENSITY RATIO ($= -\log_{10} T_{\lambda} / -\log_{10} T_{460}$) [*]		DIFFERENCE, IN PER CENT OF VALUE FOR FIG. 3
	FIG. 3	FIG. 4	
235	5.98	7.32	+22
254	2.76	3.35	+21
271	3.03	3.08	+ 2
307	1.45	1.60	+10
343	2.25	2.78	+24
370	1.88	3.10	+65
395	3.60	3.80	+ 6
418	8.77	4.69	-47
430	5.51	3.50	-36
442	2.17	1.86	-14
460	1.000	1.000	0
510	0.465	0.432	- 7

^{*}Numerator and denominator represent the optical densities at wave lengths λ and 460 $m\mu$, respectively.

the compound of hemoglobin, as is shown by the nature of the resulting modifications observed in the absorption spectrum. Therefore we conclude that the oxyhemoglobin normally present in blood is not identical with the compound formed by oxygenating a solution of purified hemoglobin in water containing a small amount of ammonium hydroxide or of sodium hydroxide.

The curves in both Figs. 3 and 4 gave evidence of a very strong absorption band, less strong in Fig. 4, with maximal absorption at 418 $m\mu$. Two other conspicuous bands appear in the ultraviolet, with maximal absorption located at or very near wave lengths 271 and 343 $m\mu$ in Fig. 3 but much less definitely indicated as to position in Fig. 4. In Fig. 4 the separation of the bands at 418 and at or near 343 $m\mu$ is masked by the absorption in the region between them. Both curves show a cut-off at about 230 $m\mu$.

Few accurate data have been published on the absorption spectrum of oxyhemoglobin in the ultraviolet and shorter wave lengths of the visible spectrum. Kayser¹⁰ refers to a number of observations made by various investigators between 1878 and 1904, but many of the data secured are unreliable or are expressed only in approximate figures. Strub²³ has published absorption curves and data for the visible region above 450 $m\mu$. Suhrmann and Kollath²⁴ show absorption curves for solutions of blood, in general shape closely similar to the curve in Fig. 3 and with the positions of the absorption bands approximately the same. But only eight values are plotted in the entire spectral range between 315 and 580 $m\mu$, so that the shape of the curve and the positions of maxima

and minima as shown can be considered only as rough approximations. Hicks and Holden⁸ have published values of the wave lengths at which maximal and minimal absorption occur in solutions of oxyhemoglobin in the ultraviolet and violet spectrum. The oxyhemoglobin was prepared by the method of Adair. In the principal band, maximal absorption was observed at 411.5 $m\mu$. Berg and Schwarzacher¹ located maximal absorption at 413.7 $m\mu$. Details of their experimental work are not given.

As shown in Fig. 3, we find that in the case of oxyhemoglobin occurring normally in human blood serum, maximal absorption in the principal absorption band occurs at 418 $m\mu$. This location is clearly indicated in a considerable number of other spectra which we have charted. Early observers, for the most part employing less accurate methods of measurement, usually assigned some value between 408 and 415 $m\mu$. In treatises on physiologic chemistry and in other recent writings, one of these earlier values usually is quoted, apparently without verification. In the absorption curves obtained by Suhrmann and Kollath, maximal absorption is indicated at about 408 $m\mu$. But the curves are only rough approximations, for reasons already stated. The few points which are plotted fall more logically on a curve showing maximal absorption at 415 or 420 $m\mu$. In the measurements by Hicks and Holden, who found maximal absorption at 411.5 $m\mu$, it is to be remembered that their results are for solutions of purified oxyhemoglobin. In view of our demonstration of the lack of identity between oxyhemoglobin normally present in blood and the compound of hemoglobin formed by oxygenating a solution of purified hemoglobin in water containing a small amount of ammonium hydroxide or sodium hydroxide, it cannot be assumed a priori that the oxyhemoglobin examined by Hicks and Holden has an absorption spectrum identical with that of oxyhemoglobin occurring normally in blood.

The average value of the absorption ratio of oxyhemoglobin at wave length 418 $m\mu$, as computed from the three curves from which Fig. 3 was calculated, is 0.000,136 gm. per sq. cm., where the concentration is in gm. per c.c. and the thickness in cm. At 427 $m\mu$ the value is 0.000,181. Using these values and the density ratios in Table II to calculate the absorption ratio at 542 $m\mu$, the wave length of maximal absorption in the β -band, the value comes out 0.00133, not in close agreement with the value 0.00110 previously found by the authors.⁶ But 0.00133 is much less reliable, being based on curves showing transmissions of 82, 85, and 88 per cent, respectively, at wave length 542. At high transmissions (T) the optical densities ($-\log_{10} T$) are small and vary rapidly with changes in T . Therefore the accuracy of the determined values of optical density and corresponding density ratios is affected to a disproportionately large degree by errors in T , the effect increasing as T approaches 100 per cent. The curve showing $T = 82$ per cent at 542 $m\mu$ gives an absorption ratio of 0.00107, in good agreement with the value found previously.

METHOD OF CORRECTING FOR ABSORPTION BY OXYHEMOGLOBIN IN DETERMINING THE ABSORPTION SPECTRUM OR CONCENTRATION OF BILIRUBIN

We have presented data which clearly show the necessity of making corrections for the absorption due to traces of oxyhemoglobin when investigating serum

bilirubin spectrophotometrically. If only the concentration of the bilirubin is to be determined, it is only necessary to find the transmission of the oxyhemoglobin at one wave length, namely, that wave length at which the transmission of the bilirubin is used in calculating its concentration. But if the nature of the investigation makes it necessary to map the absorption spectrum of the bilirubin, the transmission of the oxyhemoglobin must be determined at a sufficient number of wave lengths to make it possible to correct the observed transmission spectrum throughout its length. In either case the transmission of the oxyhemoglobin at any wave length shorter than about 520 or 530 $m\mu$ must be calculated from its concentration, since the bilirubin interferes with direct measurement of the oxyhemoglobin transmission. The two methods of estimating traces of oxyhemoglobin have been presented and discussed. The procedure to be followed in making the corrections will be made clear by the following illustration:

In Fig. 2, the portion of the curve labeled "serum" lying between wave lengths 340 and 580 $m\mu$ is the typical uncorrected curve for serum which contains an abnormally high concentration of bilirubin. The hemoglobin concentration (C) was estimated to be 0.000,17 gm. per c.c., an amount too small to be detected by the unaided eye. The thickness (L) of serum was 0.0772 cm. The absorption ratio (A) of oxyhemoglobin at 427 $m\mu$ (the choice of wave length is arbitrary, providing we use the proper absorption ratio) is 0.000,181 gm. per sq. cm. Therefore at this wave length the transmission T_{427} is found by substitution in Equation (1), which gives

$$0.000,17 = -\frac{0.000,181}{0.0772} \log_{10} T_{427},$$

from which $T_{427} = 0.846$ or 84.6 per cent. The transmissions at other wave lengths are found from this value by the use of Table II. For example, at 380 $m\mu$ we have, from the table,

$$\frac{-\log T_{380}}{-\log T_{427}} = 0.315.$$

Since $T_{427} = 0.846$, this gives $T_{380} = 0.949$ or 94.9 per cent. Plotting this value and others similarly found, the oxyhemoglobin curve shown in the figure is drawn. To correct the observed spectrum (curve marked "serum"), the transmission at each of a number of different wave lengths is divided by the corresponding transmission shown by the oxyhemoglobin curve. For example, at 430 $m\mu$ the respective transmissions are 50.3 and 87.1 per cent, therefore the corrected value is 50.3/87.1 or 57.7 per cent. This value and others similarly determined are plotted to obtain the dotted curve labeled "serum minus oxyhemoglobin," which represents the corrected bilirubin spectrum between wave lengths 340 and 580 $m\mu$.

In case the oxyhemoglobin transmission is first calculated for some wave length, λ_1 , other than 427 $m\mu$, the density ratios in Table II cannot be used. Instead, new values are found by dividing each tabulated value by the value given for λ_1 . For example, if λ_1 is 418 $m\mu$, the density ratio with respect to it will be, at 400 $m\mu$, 0.664/1.33 or 0.499; at 450 $m\mu$ it will be 0.194/1.33 or 0.146; and so on.

If it is desired only to find the concentration of the bilirubin, it is unnecessary to determine the entire oxyhemoglobin curve. Only one value of transmission is needed. For example, if the transmission of the serum is determined at wave length 410, it is necessary to know the transmission of the oxyhemoglobin only at that wave length. The method of finding it is illustrated above. The correction is made by dividing the observed transmission of the serum at 410 $m\mu$ by the corresponding transmission of the oxyhemoglobin. The corrected value is used in calculating the bilirubin concentration by means of Equation (1).

Such corrections may seem somewhat laborious. But if serum bilirubin is to be studied spectrophotometrically, either the corrections must be made or it must be shown that oxyhemoglobin is not present in amount sufficient to alter the spectrum significantly. So far as oxyhemoglobin in serum is concerned, the unaided eye is incapable of deciding whether the serum is spectrally "clear" or not.

DETERMINATION OF CONCENTRATION OF BILIRUBIN

The concentration of bilirubin is found by substituting in Equation (1) the values of A, L, and T. It is best to determine the transmission T at some wave length in the most prominent absorption band, preferably at or near the wave length at which maximal absorption occurs. In the case of bilirubin in human blood serum, we have found this wave length to be approximately 453 $m\mu$. The exact value is immaterial, since the absorption band is broad and the transmission near the minimum varies but little over a considerable spectral range (Figs. 1 and 2). Minimal transmission in the narrower oxyhemoglobin band is at 418 $m\mu$. Therefore the error due to absorption by traces of oxyhemoglobin may be largely avoided by measuring the transmission at 450 or 460 $m\mu$, since at these wave lengths the absorption by oxyhemoglobin is very much less than at 418 $m\mu$ (Fig. 3).

In Table IV are shown the values of the absorption ratios of bilirubin at wave lengths 450 and 460 $m\mu$, calculated from transmission curves obtained in our investigations of the two types of bilirubin in human blood serum and in our observations on the development of obstructive jaundice in the dog. All the serums were undiluted when examined, except the second, fifth, and sixth, which were diluted to one-third the concentration shown in the table by adding 0.1 per cent sodium carbonate solution.

The serums were prepared as follows: The blood was carefully drawn and placed in an ice box until clotted, after which the clot was loosened and the serum obtained by centrifuging. The serum was returned at once to the ice box or put in a Dewar flask containing ice at 0° C., where it was left until its spectrophotometric examination, which was made within a few hours. Since the serum was kept in the dark and at 0° or a little above, "fading" probably was inappreciable.

In averaging the values of the absorption ratios (A, Table IV), each was assigned a weight proportional to the reciprocal of the error in A resulting from an error of 1 per cent transmission in determining the transmission. Such

error in A becomes progressively larger as the transmission approaches 0 per cent or 100 per cent and is smallest in the range from about 20 per cent to 60 per cent.

The individual values of A in Table IV admittedly vary too much to warrant regarding the averages as highly accurate. To a small extent the variations are due to inaccuracy of the spectrophotometric determinations. But careful consideration has led us to conclude that the variations are due mostly to errors in the values of bilirubin concentration C as determined by the van den Bergh method. In spite of the large average deviations, the average values of A obtained from blood serum of man and of dog are in close agreement. The differences (3.5 and 1.8 per cent) are too small to be considered as other than experimental. The values for the two kinds of serum probably are identical. Also, for each serum the average values at wave lengths 450 and 460 $m\mu$ are very nearly equal. For either wave length and for the serum either of man or of dog we may consider 11.3×10^{-6} or 0.000,011,3 gm. per sq. cm. as the correct value of the absorption ratio of bilirubin. This agrees closely with the value 11.4×10^{-6} found by Cutten, Emerson and Woodruff⁷ for bilirubin dissolved in chloroform, at wave length 450 $m\mu$.

It is recommended that in determining the concentration of bilirubin in blood serum the transmission be observed at wave length 450 or 460 $m\mu$. The latter is preferable, since at this wave length oxyhemoglobin absorbs a little less radiation than at 450 $m\mu$. The absorption ratio for oxyhemoglobin at 460 $m\mu$, calculated from Table II and the value 0.000,136 at 418 $m\mu$, is 0.001,20 gm. per sq. cm.

TABLE IV

VALUES OF THE ABSORPTION RATIOS (A_{450} AND A_{460}) OF BILIRUBIN IN BLOOD SERUM, AT WAVE LENGTHS 450 AND 460 $m\mu$. C IS THE CONCENTRATION OF BILIRUBIN AS DETERMINED BY THE VAN DEN BERGH METHOD

HUMAN SERUM			DOG SERUM		
C	A_{450}	A_{460}	C	A_{450}	A_{460}
(mg. per 100 c.c.)	(gm./cm. ²)	(gm./cm. ²)	(mg. per 100 c.c.)	(gm./cm. ²)	(gm./cm. ²)
1.7	8.5×10^{-6}	8.6×10^{-6}	1.6	5.0×10^{-6}	4.8×10^{-6}
9.8	13.7	14.0	2.0	5.1	5.1
4.3	11.6	11.5	4.5	14.5	14.4
4.5	11.5	11.4	5.0	10.4	10.5
15.0	9.1	9.1	6.0	9.8	9.7
15.0	8.5	8.5	8.1	17.1	16.8
22.3	11.8	12.4	9.2	17.5	17.3
23.7	16.8	17.1			
Weighted average	11.1×10^{-6}	11.2×10^{-6}		11.5×10^{-6}	11.4×10^{-6}
Average deviation	2.1×10^{-6}	2.2×10^{-6}		4.3×10^{-6}	4.2×10^{-6}
Weighted average of all values of $A_{450} = 11.3 \times 10^{-6}$ gm. per cm. ²					
Weighted average of all values of $A_{460} = 11.3 \times 10^{-6}$ gm. per cm. ²					

SUMMARY AND CONCLUSIONS

The ultraviolet and visible absorption spectrum of bilirubin in blood serum was investigated by means of a quartz spectrograph and rotating sector photometer, with an under-water spark as source of radiation. Data were obtained

on the visibility of small amounts of oxyhemoglobin in water and in blood serum, and on the effects of traces of this pigment in spectrophotometric investigations of the components of serum, particularly bilirubin. Characteristic spectra of serum bilirubin, of serum oxyhemoglobin and of an oxygenated solution of purified hemoglobin in alkalized water are shown and discussed. Some constants of the absorption spectra of serum oxyhemoglobin and of serum bilirubin are evaluated. A method of calculating the spectrum of a small amount of oxyhemoglobin in serum and of correcting the observed spectrum is described. The principal conclusions are as follows:

1. In blood serum, traces of oxyhemoglobin not detectable by the unaided eye may absorb considerable amounts of radiation, modifying the absorption spectrum greatly.

2. In spectrophotometric investigations of bilirubin in blood serum, corrections must be made for absorption of radiation by traces of oxyhemoglobin, even in serums which appear "clear," unless it can be shown that such absorption is negligible.

3. In correcting the absorption spectrum of blood serum or of other body fluid for the absorption due to oxyhemoglobin, it is necessary to determine the concentration of oxyhemoglobin and then calculate its transmission (a) at various wave lengths, in case the nature of the spectrum is in question; or (b) at one particular wave length only, in case only the bilirubin concentration is required.

4. The most prominent absorption band of serum bilirubin is very broad. In human serum, minimal transmission in this band occurs at or near $453\text{ m}\mu$. Minimal transmission in the most prominent absorption band of oxyhemoglobin in serum is at $418\text{ m}\mu$.

5. The oxyhemoglobin normally present in blood is not identical with the compound formed by oxygenating a solution of purified hemoglobin in water containing a small amount of ammonium hydroxide or of sodium hydroxide.

6. Serum oxyhemoglobin has the following absorption ratios, in gm. per sq. cm.: at $418\text{ m}\mu$, 13.6×10^{-5} ; at $427\text{ m}\mu$, 18.1×10^{-5} ; at $460\text{ m}\mu$, 12.0×10^{-4} . The absorption ratios of serum bilirubin at 450 and $460\text{ m}\mu$ are very nearly equal, both having approximately the value 11.3×10^{-6} gm. per sq. cm., both in the serum of man and of dog.

7. It is recommended that in spectrophotometric determinations of the concentration of bilirubin in blood serum, the transmission be observed at wave length $460\text{ m}\mu$.

REFERENCES

1. Berg, F. R., and Schwarzacher, W.: Die Lage des Violettstreifens bei Oxy- und Kohlenoxydhämoglobin, *Ztschr. f. physiol. Chem.* **190**: 184, 1930.
2. Bollman, J. L., Sheard, Charles, and Mann, F. C.: Absorption of Bile Pigment From Intestine, *Am. J. Physiol.* **78**: 658, 1926.
3. Bollman, J. L., Sheard, Charles, and Mann, F. C.: An Experimental Study of Obstructive Jaundice With Particular Reference to the Initial Bilirubinemia, *Am. J. Physiol.* **80**: 461, 1927.
4. Bollman, J. L., Sheard, Charles, and Mann, F. C.: The Bilirubin Content of the Blood Following Injections of Chlorophyll, *Am. J. Physiol.* **82**: 239, 1927.
5. Cutten, Claire, Emerson, Edith E., and Woodruff, Warriner: The Fetus Index. Spectrophotometric and Quantitative Studies, *Arch. Int. Med.* **41**: 428, 1928.

6. Davis, G. E., and Sheard, Charles: Spectrophotometric Determination of Hemoglobin, *Arch. Int. Med.* **40**: 226, 1927.
7. Davis, G. E., and Sheard, Charles: Ultraviolet Spectrophotometry: Comments on Apparatus and Experimental Methods, *J. Op. Soc. Am.* **21**: 47, 1931.
8. Hicks, C. S., and Holden, H. F.: The Absorption of Ultra-violet Light by Oxyhaemoglobin and by Some of Its Derivatives, *Australian J. Exper. Biol. & Med. Sc.* **6**: 174, 1929.
9. Howe, H. E.: On a Modification of the Hilger Sector Photometer for Measuring Ultra-violet Absorption and Its Application in the Case of Certain Derivatives of Fluoran, *Physical Rev. Series II* **8**: 674, 1916.
10. Kayser, H.: *Handbuch der Spectroscopie* **4**: 147, 1908.
11. Lewis, S. J.: The Ultra-violet Absorption Spectra and the Optical Rotation of the Proteins of Blood Sera, *Proc. Roy. Soc., London, Series B* **93**: 178, 1922.
12. Magath, T. B., and Sheard, Charles: Spectrophotometric Analysis of Blood Serum in Normal and Pathologic Conditions, *Arch. Int. Med.* **39**: 214, 1927.
13. Mann, F. C., Sheard, Charles, and Bollman, J. L.: Studies on the Physiology of the Liver. XI. The Extrahepatic Formation of Bilirubin, *Am. J. Physiol.* **74**: 49, 1925.
14. Mann, F. C., Sheard, Charles, Bollman, J. L., and Baldes, E. J.: The Site of the Formation of Bilirubin, *Am. J. Physiol.* **74**: 497, 1925.
15. Mann, F. C., Sheard, Charles, Bollman, J. L., and Baldes, E. J.: Formation of Bile Pigment from Hemoglobin, *Am. J. Physiol.* **76**: 306, 1926.
16. Mann, F. C., Sheard, Charles, Bollman, J. L., and Baldes, E. J.: Studies on Physiology of Liver; Liver as Site of Bilirubin Formation, *Am. J. Physiol.* **77**: 219, 1926.
17. Mann, F. C., Sheard, Charles, and Bollman, J. L.: Evaluation of Relative Amounts of Bilirubin Formed in Liver, Spleen and Bone Marrow, *Am. J. Physiol.* **78**: 384, 1926.
18. Rahier, C.: Le dosage de la bilirubine par une méthode spectrophotométrique, *Compt. rend. Soc. de biol.* **101**: 1172, 1929.
19. Sheard, Charles, Baldes, E. J., Mann, F. C., and Bollman, J. L.: Spectrophotometric Determinations of Bilirubin, *Am. J. Physiol.* **76**: 577, 1926.
20. Sheard, Charles, Mann, F. C., and Bollman, J. L.: Spectrophotometric Determinations of Purified Bilirubin, *Am. J. Physiol.* **81**: 774, 1927.
21. Stenström, Wilhelm, and Reinhard, Melvin: Ultraviolet Absorption Spectra of Blood Sera in Relation to Infectious Diseases and to Cancer, *Am. J. Cancer* **9**: 394, 1925.
22. Stenström, Wilhelm, and Reinhard, Melvin: Ultra-violet Absorption Spectra of Blood Serum and Certain Amino Acids, *J. Biol. Chem.* **66**: 819, 1925.
23. Strub, J.: *Ztschr. f. Photographie, Photophysik u. Photochemie* **24**: 97, 1926.
24. Suhrmann, R., and Kollath, Werner: Quantitative Messungen im sichtbaren und ultravioletten Absorptionsspektrum des Blutes und seiner Bestandteile, *Biochem. Ztschr.* **184**: 216, 1927.

THE ABSORPTION SPECTRA OF THE DIRECT AND INDIRECT REACTING TYPES OF SERUM BILIRUBIN*

GEORGE E. DAVIS,† PH.D., AND
CHARLES SHEARD, PH.D., ROCHESTER, MINN.

MANY attempts have been made to explain why serum bilirubin shows two principal types (direct and indirect) of reaction when various samples are subjected to the van den Bergh test with sulphanilic acid and sodium nitrite. In obstructive jaundice the reaction is usually prompt or "direct"; in hemolytic jaundice, typically delayed or "indirect." It is not possible in this paper to review the numerous researches bearing upon such differences in reaction or the various theories proposed to account for them. Hunter⁶ has given a brief review of the more important investigations and theories to 1930, together with a summary of the principal chemical, physical and physiologic evidence concerning the existence of two types of bilirubin. We shall confine ourselves to a brief consideration of the investigations involving spectrophotometric methods.

Magath and Sheard⁹ made spectrophotometric analyses of normal human blood serums and of serums of patients who had malaria, pernicious anemia, hemolytic jaundice and obstructive jaundice. Values of transmission in the spectral range from 700 to 430 $m\mu$ were obtained by means of a visual spectrophotometer, the Keuffel and Esser "color analyzer." No differences were found in the types of spectrophotometric curves obtained from diluted serums exhibiting the direct and indirect types of reaction. It was concluded that if the bilirubin contained in blood serums differs, it cannot be shown spectrophotometrically. This conclusion was applicable only to the visible spectrum in the range in which the observations were made (700-430 $m\mu$) and to serums diluted with a mixture of alcohol and acetone.

Heilmeyer and Krebs³ prepared diazotized alcoholic extracts of serum containing the direct and indirect types of bilirubin, using the method of Thannhauser and Andersen. They compared the absorption spectra of these extracts in the visual range from 650 to 470 $m\mu$, using a König-Martens spectrophotometer. They considered the spectrophotometric curves to be almost identical in shape, although certain definite differences were evident, particularly in the spectral region between 575 and 470 $m\mu$ where the curves diverge greatly.

*From the Division of Physics and Biophysical Research, The Mayo Foundation.

Received for publication, February 14, 1937.

†Formerly a fellow in the Mayo Foundation. Now associate in the Department of Physics, Duquesne University, Pittsburgh, Pa.

Furthermore, the types of curve obtained also differ from the curve for azo-pigment in chloroform and alcohol, formed by diazotizing purified bilirubin. The authors concluded from the similarity of the spectra that the two forms of bilirubin discussed by van den Bergh give identical azopigments and that differences in the bilirubin molecule are not responsible for variations in the diazo reaction.

Müller and Engel¹⁰ stated that their experiments with serums in cases of icterus confirm the conclusion of Magath and Sheard that the absorption spectra of direct and indirect reacting bilirubins are the same. They stated that the spectra agree completely, and also agree with the spectrum of normal serum to which bilirubin has been added artificially. The spectrophotometric evidence upon which this conclusion is based is not presented. They also prepared diazotized solutions (method of Thannhauser and Andersen) of bilirubin in chloroform, of normal serum with bilirubin added artificially, and of direct- and indirect-reacting serums in cases of icterus. Absorption spectra of these solutions were obtained in the spectral region between 650 and 470 $m\mu$, using a König-Martens spectrophotometer. It is shown that the curves of spectral absorption can be made to coincide almost exactly from 650 $m\mu$ to beyond the wave length of maximal absorption, which is approximately 575 $m\mu$. The four curves begin to diverge at about 550 $m\mu$ and spread farther and farther apart as wave length 470 $m\mu$ is approached. According to Müller and Engel, these differences are due to the presence of different amounts of uncoupled bilirubin. This explanation is based upon the statement of Fischer and Barrenscheen (1921) that the coupling in the diazo reaction is never complete. The authors concluded that the bilirubins in direct- and indirect-coupled serums exhibit no spectral differences or changes in crystalline form. It is to be noted, however, that the spectrophotometric evidence as presented does not warrant this conclusion being applied to the bilirubins as they occur naturally in blood serum, since it is not proved that any chemical or physical differences which may exist are not destroyed during preparation of the diazotized solutions. The conclusion must be taken to apply only to the bilirubins after they have been changed to the azo-pigments.

Heilmeyer and Toop⁴ obtained a considerable number of visual absorption spectra of pigments occurring in blood serum, including bilirubin in the following natural and artificially prepared solutions: natural serum bilirubin produced by hemolysis in vivo, induced by injection of distilled water; human serum with bilirubin injected in vivo; purified bilirubin in various solvents, including blood serum; human bile; serums from patients with icterus, icterus catarrh, and hemolytic icterus. The spectra are represented by straight-line curves only, with the lower limit at 420 $m\mu$ or above. The authors do not draw any conclusions concerning the possibility of distinguishing between the two van den Bergh types of bilirubin spectrophotometrically. It is unlikely that any general differentiation could be made from the curves they have presented.

In these researches, the absorption spectra were examined only in the visible spectrum, the lower limits of wave length being: Magath and Sheard, 430 $m\mu$; Heilmeyer and Krebs, and Müller and Engel, 470 $m\mu$; Heilmeyer and Toop,

420 $m\mu$ or above. These observations cover approximately only half of the broad characteristic absorption band of bilirubin, which extends well into the ultra-violet. In the present investigations we have measured the absorption of the two types of serum bilirubin over the entire band, examining the spectra of various samples more critically and comparing the spectra of the two types of bilirubin with greater quantitative accuracy than has been evidenced in previous investigations.

EXPERIMENTAL PROCEDURE

The absorption spectra of a number of samples of human serum in cases of icterus were obtained. Some of the serums gave the direct, some the indirect, type of van den Bergh reaction. It should be borne in mind that serums containing direct-reacting serum bilirubin also may and usually do contain a varying amount of indirect-reacting bilirubin. Since it is impossible to separate these two types, curves plotted from determinations made on the direct type represent results obtained on mixtures of this type and the indirect type. Hence in referring to spectrophotometric data obtained from direct-reacting serums, it is to be understood that all the bilirubin in these serums was not of the direct-reacting type.

The spectra were represented by plotting transmissions against wave lengths. The experimental procedure was as follows: About 10 c.c. of blood was obtained from a patient known to have pronounced jaundice, care being taken to avoid hemolysis so far as possible. The blood was at once put into a refrigerator to clot. After about one hour the clot was carefully loosened and the blood was centrifuged. The serum was returned to the refrigerator or put into a Dewar flask containing ice at 0° C., where it was kept until examined spectrophotometrically. An estimation of the concentration of bilirubin was made on a portion of the serum by the van den Bergh method.

The spectrophotometric examinations of the serums were made by means of a large quartz spectrograph (photographic plates 2.6 by 10 inches) and a rotating sector photometer, employing an under-water spark between tungsten terminals as the source of radiation. The instruments were assembled and used in the usual manner, the method being essentially as first described by Howe.⁵ The electrical circuits, quartz cells and container for the under-water spark have been described elsewhere by the authors.¹

Spectroscopic examination showed that most blood serums, however carefully prepared, contained oxyhemoglobin sufficient in amount to affect the absorption spectrum even though the serum appeared to the unaided eye to be almost or quite free from red color. Bilirubin could not be removed from the serum with the assurance that the spectral transmission of the other components would remain unchanged. Because of this fact we could not use bilirubin-free serum as the comparison fluid. Therefore distilled water was used instead and correction was made for the absorption in the oxyhemoglobin when traces of that pigment were present. The corrections involved the following steps: (1) estimation of the concentration of oxyhemoglobin in the serum; (2) calculation of the curve of spectral transmission of the oxyhemoglobin alone; (3) correction of the observed transmission curve of the serum by dividing the transmission,

at each of a sufficient number of wave lengths, by the corresponding transmission of the oxyhemoglobin, as shown by the calculated curve. The details of this method of correcting for traces of oxyhemoglobin have been described previously.²

The absorption spectra of six serums giving the direct van den Bergh reaction were determined. We were able to obtain only a few good serums showing the indirect reaction and having a bilirubin concentration high enough to give an absorption spectrum relatively little affected by absorption in components of the serum other than traces of oxyhemoglobin. Reliable transmission curves were obtained for three serums which gave the indirect reaction. Six of the nine serums were examined without dilution or the addition of any substance. Three serums were diluted to one part in three by adding a 0.1 per cent solution of sodium carbonate. In every case but one the serums were examined in a quartz cell 0.77 mm. thick; in the one case the thickness was 5.84 mm. The absorption spectra of the serums were represented by plotting transmissions against wave lengths.

ABSORPTION SPECTRA OF SERUM BILIRUBIN

Four of the spectra are shown in Fig. 1 and illustrate the typical characteristics and the variations obtained. The concentrations of bilirubin were determined by the van den Bergh method: the data are given in the legend accompanying the curves.

Before comparing the curves, it is to be noted that the absorption band at 280 $m\mu$, Curve 2, is not characteristic of bilirubin but is due to serum proteins, as shown by Lewis.⁸ Although Curves 1, 3, and 4 are not extended far enough to show this band, nevertheless the shapes of the curves below 330 or 340 $m\mu$ are affected by it. For this reason the curves will be compared only in the spectral region above about 340 $m\mu$.

Marked variations in the spectrophotometric transmissions, even for bilirubins of the same type, are quite apparent. Significant differences in absorption are shown by the major contours of the curves. For example, in Curves 2, 3 and 4, representing nearly equal concentrations of bilirubin (Curve 2, direct; Curves 3 and 4, indirect), the transmissions are very nearly equal at wave length 495 $m\mu$ but differ widely throughout most of the spectral range of the measurements. Curves 2 and 4 cross at 495 $m\mu$ and practically coincide from 335 to 360 $m\mu$; but the transmission shown in Curve 4 is about 29 per cent greater at 410 $m\mu$ and 16 per cent less at 530 $m\mu$. That these differences are not necessarily chiefly due to the difference in type of bilirubin, however, is shown by the fact that similar large differences in transmission, varying in sense (positive or negative) one or more times along the spectral ranges represented, are seen in comparing Curves 2 and 3 (direct and indirect) and Curves 3 and 4 (both indirect). The absorption spectra of bilirubins of the same van den Bergh type often differ as markedly as they differ from the spectra of bilirubins unlike them in type. The differences are commonly many times larger than the greatest possible experimental error of the measurements.

Distinct minor variations in the spectra of both types of bilirubin also were observed, and they are illustrated in the curves of Fig. 1. These variations

are doubtless genuine, since the experimental errors are generally too small to be largely responsible for them. The observed values of transmission, uncorrected for absorption due to oxyhemoglobin, are correct to within approximately 1 per cent transmission. Occasionally the errors in the corrected curves may be greater in cases in which the corrections are large, but they should seldom exceed 2 per cent. We should not expect the errors to vary abruptly in short spectral ranges in such a manner as to cause the type of wavy or steplike sections shown in certain portions of the curves. Similar variations in or near the absorption band at $280\text{ m}\mu$, were observed by Lewis⁷ in the absorption spectra of blood serums, and later⁸ in the spectra of euglobulin, pseudoglobulin and albumin. The curves were described by Lewis as having steplike irregularities which varied with the pathologic conditions of the subjects (human) from whom the serums were obtained.

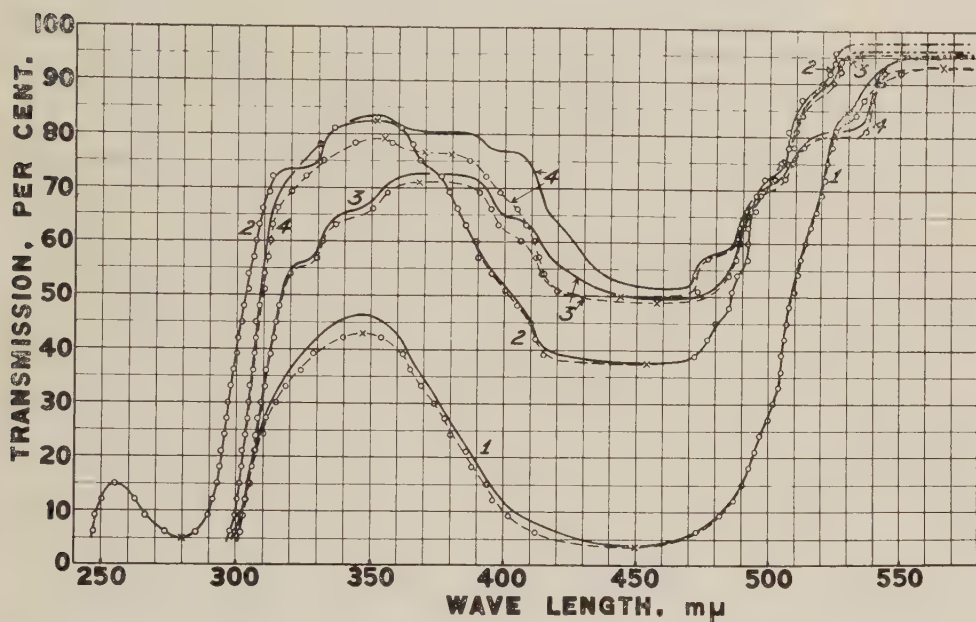


Fig. 1.—Transmission curves of blood serum from patients. These curves are typical for serum bilirubin in the spectral region above about $340\text{ m}\mu$. Dotted curves, observed transmissions; full line curves, after correction for absorption by traces of oxyhemoglobin. Types of reaction and concentrations of bilirubin (mg. per 100 c.c.) as determined by the van den Bergh method: Curve 1, direct, 22.3; Curve 2, direct, 5.0 (original serum 15.0, here reduced by dilution); Curve 3, indirect, 4.5; Curve 4, indirect, 4.3. Thickness of spectroscopic cell, 0.77 mm. in each case.

These minor variations in the spectra undoubtedly are due mainly to differences in chemical composition, either in the bile pigment or in the other components of the serum, or in both. Since water was used as the comparison substance, the spectral curves show the absorption not only of bilirubin but of all the other serum components except oxyhemoglobin. If it had been possible in each case to remove the bilirubin from some of the serum without affecting the other components, and to use this bilirubin-free serum as the comparison substance, the spectral curves would show only the absorption by the bilirubin.

It is evident that any differences which may occur in the absorption spectra, as between the direct and indirect reaction types of bilirubin, are not revealed on gross examination of the curves but are masked by the individual variations which occur in the spectra of both types. Therefore, in order more adequately to analyze and compare the spectra, we have recorded and averaged in Table I the wave lengths of maximal absorption in the typical bilirubin band, the wave lengths of minimal absorption adjacent to this band on the short-wave side, and the values of six ratios of optical densities. The optical density D is the negative logarithm of the transmission, to base 10. The ratio of the optical densities corresponding to any two wave lengths in the absorption spectrum of a substance is a constant, regardless of the concentration or the thickness of solution examined. Hence in comparing the spectra of two substances, any differences in corresponding ratios of optical density may be considered evidence that the substances are not identical. All of the ratios here given involve densities in the characteristic absorption band of bilirubin.

The values of the wave length of maximal absorption do not vary greatly, considering that the absorption band is broad and nearly "flat" in that region, making it more difficult to determine the position of the maximal accurately. The average wave length of maximal absorption for the indirect bilirubins, 459.3 m μ , is about 10 m μ greater than the value for the direct bilirubins, 449.5 m μ .

The wave lengths of minimal absorption adjacent to the bilirubin band vary somewhat more widely from sample to sample. The difference of about 8 m μ between the average values for the two types of pigment is no greater than we should expect from the individual variations. It is to be noted, however, that the value for the indirect bilirubins is the higher, as is the case for the values at maximal absorption.

The ratios of optical densities shown in the table must be examined carefully in deciding whether there are any real differences between the average

TABLE I
CONSTANTS OF THE ABSORPTION SPECTRA OF SAMPLES OF DIRECT AND INDIRECT SERUM BILIRUBIN

TYPE OF VAN DEN BERGH REACTION	CONCENTRA- TION OF BILIRUBIN, MG. PER 100 C.C.	WAVE LENGTH OF MAXIMUM ABSORPTION, M μ .	WAVE LENGTH OF MINIMUM ABSORPTION M μ .	RATIOS OF OPTICAL DENSITIES (D) ^a					
				D _{min}	D ₄₀₀	D ₄₉₀	D ₅₁₀	D ₄₀₀	D ₅₁₀
				D _{max}	D _{max}	D _{max}	D _{max}	D ₄₀₀	D ₄₀₀
Direct	23.7	448	347	0.27	0.65	0.57	0.23	0.88	0.34
Direct	22.3	450	347	0.23	0.64	0.56	0.19	0.88	0.29
Direct	5.0	455	352	0.26	0.66	0.66	0.24	1.01	0.37
Direct	5.0	454	352	0.19	0.68	0.60	0.17	0.89	0.25
Direct	3.3	450	362	0.46	0.59	0.61	0.36	1.04	0.61
Direct	1.7	440	361	0.54	0.64	0.80	0.45	1.25	0.70
Indirect	4.3	460	354	0.27	0.40	0.65	0.40	1.62	0.99
Indirect	1.0	458	(?)†	0.35	0.35	0.82	0.50	2.34	1.43
Indirect	4.5	460	372	0.46	0.62	0.66	0.29	1.07	0.47
Average, directs		449.5	355.2	0.32	0.64	0.63	0.27	0.99	0.43
Average, indirects		459.3	363.0	0.36	0.46	0.71	0.40	1.68	0.96
Average, all		452.8	357.1	0.34	0.58	0.66	0.31	1.22	0.61

^aD_{max} and D_{min} are the optical densities at the wave lengths of maximal and minimal absorption, respectively. The numerical subscripts 400, 490, and 510 indicate spectral positions at these wave lengths (m μ).

†Value not definitely indicated by the curve.

values for the direct and indirect-reacting types of bilirubin. Considering the magnitude of the variation in each ratio from sample to sample of serum, the differences in the average values seem significant except for $D_{\min}/D_{\max}$ and $D_{490}/D_{\max}$. The data therefore indicate that, on the average, $D_{400}/D_{\max}$ is greater, while $D_{510}/D_{\max}$, D_{490}/D_{400} and D_{510}/D_{400} are less, for the direct reaction type of bilirubin than for the indirect type. Considering that the reciprocal of the first quantity is less for the direct types, these results mean that the absorption at 400 $m\mu$, as compared to the absorptions at the maximum and at 490 and 510 $m\mu$, is greater for the direct reaction type than for the indirect. The absorption at the maximum, as compared to that at 510 (and possibly at 490) $m\mu$, also is greater for the direct types. In speaking of transmissions, these statements should be reversed. These data are consistent, indicating that the typical bilirubin absorption band lies farther toward the shorter wave lengths when the reaction is direct than when it is indirect. This is shown also by the fact that the average wave length of maximal absorption is about 10 $m\mu$ less for the direct reaction pigment. The average wave length of minimal absorption adjacent to the band appears to be similarly shifted, as has been noted. While these results are clearly indicated, they are not conclusive, since the number of samples of serum examined is small and the variations in any one absorption ratio are relatively large. A larger number of samples should be examined. The average values of the absorption ratios shown in Table I are approximate characteristic constants of the spectra of the two forms of serum bilirubin.

While it cannot be stated positively that the presence of indirect-reacting bilirubin in serum containing direct-reacting pigment changes to any marked degree the type of the curve, it seems likely that the difference in the absorption spectra between direct and indirect-reacting bilirubin is actually greater than is here indicated, and that if a serum containing only direct-reacting bilirubin were obtained, the maximal absorption would be obtained at a wave length shorter than we have found. It will be appreciated that the problem is complicated by this situation and that, in reality, we were comparing the spectra of indirect-reacting bilirubin (in serum) with a serum containing a mixture of direct- and indirect-reacting bilirubin in which the proportion of the two was unknown.

SUMMARY

The absorption spectra of a number of samples of serum bilirubin (showing the direct and indirect types of van den Bergh reaction, respectively) were obtained by means of the quartz spectrograph and a sector photometer. Typical spectral transmission curves are shown and discussed. The absorption spectra of different specimens vary markedly, even for bilirubins of the same type. These variations tend to mask any differences in spectra which may be due to a difference in type of pigment. By comparing various absorption ratios and the wave lengths of maximal and minimal absorption, it is shown that the spectrum of serum containing direct-reacting bilirubin differs from that of the indirect type in that its characteristic absorption band is shifted about 10 $m\mu$ farther toward the shorter wave lengths. A similar difference of about 8 $m\mu$ is indi-

cated for the wave lengths of minimal absorption adjacent to the band. In serum, the average values of the wave lengths of maximal absorption were found to be approximately 449 $m\mu$ for the direct-reacting serum bilirubins and 459 $m\mu$ for the indirect type; the minimal absorption in the ultraviolet region of the spectrum of serum bilirubin occurred at about 355 $m\mu$ for the direct type and at 363 $m\mu$ for the indirect type. These data suggest that the two types of serum bilirubin may be distinguished spectrophotometrically if methods adequate for such determinations in the violet and ultraviolet portions of the spectrum are employed. However, the problem is complicated by the presence of indirect-reacting bilirubin in serum containing direct-reacting pigment.

REFERENCES

1. Davis, G. E., and Sheard, Charles: Ultraviolet Spectrophotometry: Comments on Apparatus and Experimental Methods, *J. Optic Soc. America* 21: 47, 1931.
2. Davis, G. E., and Sheard, Charles: The Spectrophotometric Investigation and Determination of Bilirubin, *J. LAB. & CLIN. MED.* 19: 593, 1934.
3. Heilmeyer, Ludwig, and Krebs, Willy: Spektrophotometrische Untersuchungen des Ehrlich-Pröscherschen Bilirubin-Azofarbstoffes und ihre praktische Anwendung, besonders zur quantitativen Bestimmung des Bilirubins im Blutserum, *Biochem. Ztschr.* 223: 352, 1930.
4. Heilmeyer, L., and Toop, H.: Klinische Farbmessungen. XII. Mitteilung. Die Spektrophotometrische Farbstoffanalyse des Blutserums mit besonderer Berücksichtigung des Bilirubin- und Carotinspektrums, *Ztschr. f. d. ges. exper. Med.* 80: 603, 1932.
5. Howe, H. E.: On a Modification of the Hilger Sector Photometer for Measuring Ultraviolet Absorption and Its Application in the Case of Certain Derivatives of Fluoran, *Physical Rev.* 8: 674, 1916.
6. Hunter, George: On the Two Types of Bilirubin Diazo-Reaction in Serum, With a Hypothesis on the Nature of the Bilirubin in the Serum From Haemolytic Jaundice, *Brit. J. Exper. Path.* 11: 415, 1930.
7. Lewis, S. J.: The Ultra-Violet Absorption Spectra of Blood Sera, *Proc. Roy. Soc. London* 89: 327, 1916.
8. Lewis, S. J.: The Ultra-Violet Absorption Spectra and the Optical Rotation of the Proteins of Blood Sera, *Proc. Roy. Soc. London* 93: 178, 1922.
9. Magath, T. B., and Sheard, Charles: Spectrophotometric Analysis of Blood Serum in Normal and Pathologic Conditions. Study I, *Arch. Int. Med.* 39: 214, 1927.
10. Müller, P., and Engel, L.: Über das Absorptionsspektrum des Bilirubins in verschiedenen Lösungsmitteln. III. Mitteilung, *Hoppe-Seyler's Ztschr.* 202: 56, 1931.

Papers in abstract:

Coefficients of Diffusion of Salt Vapors in the Bunsen Flame. *Proceedings of the Iowa Academy of Science*, 29:155. 1922.

An Attempt To Detect a Predicted Pressure Effect in Gases. *Proceedings of the Iowa Academy of Science*, 32:378-379. 1925.

A Simple Derivation of the Equation for Mean Free Path. *Proceedings of the Iowa Academy of Science*, 33:250. 1926.

A New Analysis of the Action of Amsler's Polar Planimeter. *Proceedings of the Iowa Academy of Science*, 33:250-251. 1926.

Correlation of Neatness and Scholarship in Physics. *Proceedings of the Iowa Academy of Science*, 34:278. 1927.

A Spectrophotometric Method of Studying Hemoglobin and Other Colored Substances in Solution. *Proceedings of the Iowa Academy of Science*, 34:279-280. 1927.

The Spectral Distribution of Erythral Effectiveness of Ultraviolet Radiation. *Proceedings of the Iowa Academy of Science*, 40:147. 1933.

